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Application for Patent

אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
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חברה רשומה בישראל
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רחובות

בעל אמצאה מכח העברה ששמה הוא Assignment
Owner, by virtue of ... of an Invention the title of which is

(בעברית) (Hebrew) מודולטורים של קולטנים ממשפחת-העל TNF/NGF, הכנתם והשימוש בהם

(באנגלית) (English) Modulators of TNF/NGF superfamily receptors, their preparation and use

מבקש בזאת כי ינתן לי עליה פטנט
hereby apply for a patent to be granted to me in respect thereof.

• בקשת חלוקה — Application of Division	• בקשת פטנט מוסף — Application for Patent Addition	• דרישה דין קדימה Priority Claim		
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For the Applicant

Henry Einav

היום 15 בחודש דצמבר שנת 1994
This 15 of December of the year 1994

לשימוש הלשכה
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MODULATORS OF TNF/NGF SUPERFAMILY RECEPTORS, THEIR PREPARATION AND USE

מודולטורים של קולטנים ממשפחת-העל TNF/NGF
הכנתם והשימוש בהם

Yeda Research and Development Company Limited
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Field of the Invention

The present invention is generally in the field of receptors belonging to the TNF/NGF superfamily of receptors and the control of their biological functions. The TNF/NGF superfamily of receptors includes receptors such as the p55 and p75 tumor necrosis factor receptors (TNF-Rs) and the FAS ligand receptor (FAS-R) and others. More specifically, the present invention concerns novel proteins which bind to the intracellular domains (IC) of the p55 and p75 TNF-Rs and the Fas-R, (these intracellular domains designated p55IC, p75IC and Fas-IC, respectively) and which novel proteins are capable of modulating the function of the p55 and p75 TNF-Rs and the Fas-R. The present invention also concerns the preparation and uses of these novel p55 and p75 TNF-R-binding proteins, and Fas-R binding proteins, referred to herein as p55IC-, p75IC- and Fas-IC- binding proteins.

Background of the Invention and Prior Art

The present application is an application for a patent of Addition to the co-pending Israel Patent Application Nos. 109632 and 111125, in which there are disclosed, among other aspects, new proteins which are capable of binding to either the intracellular domain of the p75 TNF-R or the intracellular domain of the p55 TNF-R (p75 IC- and p55-IC-binding proteins respectively); as well as the fact that the p55 IC and FAS-IC are capable of self-association and contain within their IC domain a region called the 'death domain' (DD) which is primarily responsible for this self-association. Thus, both the p55IC and FAS-IC as well as the p55IC and FAS-IC death domains (p55DD and FAS-DD, respectively) also represent p55IC- and FAS-IC- binding proteins capable of mediating the function of these receptors. The present invention concerns proteins which can bind to the intracellular domains of receptors belonging to the TNF/NGF family in general, and proteins capable of binding to the p55IC, p75IC and FAS-IC in particular. These IC-binding proteins include new proteins as well as portions, e.g. the p55IC, p55DD, FAS-IC

and FAS-DD, of intracellular domains of the various TNF/NGF receptors that are capable of binding TNF/NGF receptor intracellular domains.

Tumor Necrosis Factor (TNF-α) and Lymphotoxin (TNF-β) (hereinafter, TNF, refers to both TNF-α and TNF-β) are multifunctional pro-inflammatory cytokines formed mainly by mononuclear phagocytes, which have many effects on cells (Wallach, D. (1986) in : Interferon 7 (Ion Gresser, ed.), pp. 83-122, Academic Press, London; and Beutler and Cerami (1987)). Both TNF-α and TNF-β initiate their effects by binding to specific cell ~~surface receptors. Some of the effects are likely to be beneficial to the organism : they may~~ destroy, for example tumor cells or virus infected cells and augment antibacterial activities of granulocytes. In this way, TNF contributes to the defense of the organism against tumors and infectious agents and contributes to the recovery from injury. Thus, TNF can be used as an anti-tumor agent in which application it binds to its receptors on the surface of tumor cells and thereby initiates the events leading to the death of the tumor cells. TNF can also be used as an anti-infectious agent.

However, both TNF-α and TNF-β also have deleterious effects. There is evidence that over-production of TNF-α can play a major pathogenic role in several diseases. Thus, effects of TNF-α, primarily on the vasculature, are now known to be a major cause for symptoms of septic shock (Tracey et al., 1986). In some diseases, TNF may cause excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia, and TNF-α was thus called cachetin. It was also described as a mediator of the damage to tissues in rheumatic diseases (Beutler and Cerami, 1987) and as a major mediator of the damage observed in graft-versus-host reactions (Piquet et al., 1987). In addition, TNF is known to be involved in the process of inflammation and in many other diseases.

Two distinct, independently expressed, receptors, the p55 and p75 TNF-Rs, which bind both TNF-α and TNF-β specifically, initiate and/or mediate the above noted biological effects of TNF. These two receptors have structurally dissimilar intracellular domains suggesting that they signal differently (See Hohmann et al., 1989; Engelmann et al., 1990; Brockhaus et al., 1990; Leotscher et al., 1990; Schall et al., 1990; Nophar et al.,

1990; Smith et al., 1990; and Heller et al., 1990). However, the cellular mechanisms, for example, the various proteins and possibly other factors, which are involved in the intracellular signaling of the p55 an p75 TNF-Rs have yet to be elucidated (In IL 109632 and as set forth also herein below, there is described for the first time, new proteins capable of binding to the p75IC and p55 IC). It is this intracellular signaling, which occurs usually after the binding of the ligand, i.e. TNF (α or β), to the receptor, that is responsible for the commencement of the cascade of reactions that ultimately result in the observed response of the cell to TNF.

As regards the above mentioned cytocidal effect of TNF, in most cells studied so far, this effect is triggered mainly by the p55 TNF-R. Antibodies against the extracellular domain (ligand binding domain) of the p55 TNF-R can themselves trigger the cytocidal effect (see EP 412486) which correlates with the effectivity of receptor cross-linking by the antibodies, believed to be the first step in the generation of the intracellular signaling process. Further, mutational studies (Brakebusch et al., 1992; Tartaglia et al., 1993) have shown that the biological function of the p55 TNF-R depends on the integrity of its intracellular domain, and accordingly it has been suggested that the initiation of intracellular signaling leading to the cytocidal effect of TNF occurs as a consequence of the association of two or more intracellular domains of the p55 TNF-R. Moreover, TNF (α and β) occurs as a homotrimer and as such has been suggested to induce intracellular signaling via the p55 TNF-R by way of its ability to bind to and to cross-link the receptor molecules, i.e. cause receptor aggregation. In IL 109632 and IL 111125 and also herein below there is described how the p55IC and p55DD can self-associate and induce, in a ligand-independent fashion, TNF-associated effects in cells.

Another member of the TNF/NGF superfamily of receptors is the FAS receptor (FAS-R) which has also been called the Fas antigen, a cell-surface protein expressed in various tissues and sharing homology with a number of cell-surface receptors including TNF-R and NGF-R. The FAS-R mediates cell death in the form of apoptosis (Itoh et al., 1991), and appears to serve as a negative selector of autoreactive T cells, i.e. during maturation of T cells, FAS-R mediates the apoptopic death of T cells recognizing self-

antigens. It has also been found that mutations in the FAS-R gene (*lpr*) cause a lymphoproliferation disorder in mice that resembles the human autoimmune disease systemic lupus erythematosus (SLE) (Watanabe-Fukunaga et al., 1992). The ligand for the FAS-R appears to be a cell-surface associated molecule carried by, amongst others, killer T cells (or cytotoxic T lymphocytes - CTLs), and hence when such CTLs contact cells carrying FAS-R, they are capable of inducing apoptopic cell death of the FAS-R-carrying cells. Further, a monoclonal antibody has been prepared that is specific for FAS-R, this monoclonal antibody being capable of inducing apoptopic cell death in cells carrying FAS-R, including mouse cells transformed by cDNA encoding human FAS-R (Itoh et al., 1991).

It has also been found that various other normal cells, besides T lymphocytes, express the FAS-R on their surface and can be killed by the triggering of this receptor. Uncontrolled induction of such a killing process is suspected to contribute to tissue damage in certain diseases, for example, the destruction of liver cells in acute hepatitis. Accordingly, finding ways to restrain the cytotoxic activity of FAS-R may have therapeutic potential.

Conversely, since it has also been found that certain malignant cells and HIV-infected cells carry the FAS-R on their surface, antibodies against FAS-R, or the FAS-R ligand, may be used to trigger the FAS-R mediated cytotoxic effects in these and thereby provide a means for combating such malignant cells or HIV-infected cells (see Itoh et al., 1991). Finding yet other ways for enhancing the cytotoxic activity of FAS-R may therefore also have therapeutic potential.

It has been a long felt need to provide a way for modulating the cellular response to TNF (α or β) and FAS-R ligand, for example, in pathological situations as mentioned above, where TNF or FAS-R ligand is over-expressed it is desirable to inhibit the TNF- or FAS-R ligand- induced cytocidal effects, while in other situations, e.g. wound healing applications, it is desirable to enhance the TNF effect, or in the case of FAS-R, in tumor cells or HIV-infected cells it is desirable to enhance the FAS-R mediated effect.

A number of approaches have been made by the present inventors (see for example, European Application Nos. EP 186833, EP 308378, EP 398327 and EP 412486)

to regulate the deleterious effects of TNF by inhibiting the binding of TNF to its receptors using anti-TNF antibodies or by using soluble TNF receptors (being essentially the soluble extracellular domains of the receptors) to compete with the binding of TNF to the cell surface-bound TNF-Rs. Further, on the basis that TNF-binding to its receptors is required for the TNF-induced cellular effects, approaches by the present inventors (see for example IL 101769 and its corresponding EPO 568925) have been made to modulate the TNF effect by modulating the activity of the TNF-Rs. Briefly, EPO 568925 (IL 101769) relates to a method of modulating signal transduction and/or cleavage in TNF-Rs whereby peptides or other molecules may interact either with the receptor itself or with effector proteins interacting with the receptor, thus modulating the normal functioning of the TNF-Rs. In EPO 568925 there is described the construction and characterization of various mutant p55 TNF-Rs, having mutations in the extracellular, transmembranal, and intracellular domains of the p55 TNF-R. In this way regions within the above domains of the p55 TNF-R were identified as being essential to the functioning of the receptor, i.e. the binding of the ligand (TNF) and the subsequent signal transduction and intracellular signaling which ultimately results in the observed TNF-effect on the cells. Further, there is also described a number of approaches to isolate and identify proteins, peptides or other factors which are capable of binding to the various regions in the above domains of the TNF-R, which proteins, peptides and other factors may be involved in regulating or modulating the activity of the TNF-R. A number of approaches for isolating and cloning the DNA sequences encoding such proteins and peptides; for constructing expression vectors for the production of these proteins and peptides; and for the preparation of antibodies or fragments thereof which interact with the TNF-R or with the above proteins and peptides that bind various regions of the TNF-R, are also set forth in EPO 568925. However, no description is made in EPO 568925 of the actual proteins and peptides which bind to the intracellular domains of the TNF-Rs (e.g. p55 TNF-R), nor is any description made of the yeast two-hybrid approach to isolate and identify such proteins or peptides which bind to the intracellular domains of TNF-Rs. Similarly, heretofore there has been no disclosure of proteins or peptides capable of binding the intracellular domain of FAS-R.

Thus, when it is desired to inhibit the effect of TNF, or the FAS-R ligand, it would be desirable to decrease the amount or the activity of TNF-Rs or FAS-R at the cell surface, while an increase in the amount or the activity of TNF-Rs or FAS-R would be desired when an enhanced TNF or FAS-R ligand effect is sought. To this end the promoters of both the p55 TNF-R and the p75 TNF-R have been sequenced, analyzed and a number of key sequence motifs have been found that are specific to various transcription regulating factors, and as such the expression of these TNF-Rs can be controlled at their promoter level, i.e. inhibition of transcription from the promoters for a decrease in the number of receptors, and an enhancement of transcription from the promoters for an increase in the number of receptors (see IL 104355 and IL 109633). Corresponding studies concerning the control of FAS-R at the level of the promoter of the FAS-R gene have yet to be reported.

Thus, prior to IL 109632 and the present invention, there have not been provided proteins which may regulate the effect of ligands belonging to the TNF/NGF superfamily, such as the TNF or FAS-R ligand effect on cells, by mediation of the intracellular signaling process, which signaling is probably governed to a large extent by the intracellular domains (ICs) of the receptors belonging to the TNF/NGF superfamily of receptors, such as those of the TNF-Rs, i.e. the p55 and p75 TNF-R intracellular domains (p55IC and p75IC, respectively), as well as the FAS-IC.

Accordingly, it is one aim of the invention to provide proteins which are capable of binding to the intracellular domains of the TNF-Rs and FAS-R, which proteins are presently believed to be involved in the intracellular signaling process initiated by the binding of TNF to its receptors, or the binding of FAS ligand to its receptor.

Another aim of the invention is to provide antagonists (e.g. antibodies) to these intracellular domain-binding proteins (IC-binding proteins) which may be used to inhibit the signaling process, when desired, when such IC-binding proteins are positive signal effectors (i.e. induce signaling), or to enhance the signaling process, when desired, when such IC-binding proteins are negative signal effectors (i.e. inhibit signaling).

Yet another aim of the invention is to use such IC-binding proteins to isolate and characterize additional proteins or factors, which may, for example, be involved further downstream in the signaling process, and/or to isolate and identify other receptors further upstream in the signaling process to which these IC-binding proteins bind (e.g. other TNF-Rs or related receptors), and hence, in whose function the IC-binding proteins are also involved.

Moreover, it is an aim of the present invention to use the above-mentioned IC-binding proteins as antigens for the preparation of polyclonal and/or monoclonal antibodies thereto. The antibodies, in turn, may be used for the purification of the new IC-binding proteins from different sources, such as cell extracts or transformed cell lines.

Furthermore, these antibodies may be used for diagnostic purposes, e.g. for identifying disorders related to abnormal functioning of cellular effects mediated by receptors belonging to the TNF/NGF receptor superfamily.

A further aim of the invention is to provide pharmaceutical compositions comprising the above IC-binding proteins, and pharmaceutical compositions comprising the IC-binding protein antagonists, for the treatment or prophylaxis of TNF-induced or FAS ligand-induced conditions, for example, such compositions can be used to enhance the TNF or FAS ligand effect or to inhibit the TNF or FAS ligand effect depending on the above noted nature of the IC-binding protein or antagonist thereof contained in the composition.

__Summary of the Invention__

In accordance with the present invention, we have found novel proteins which are capable of binding to either the intracellular domain of the p55 TNF-R (the p55IC-binding proteins), of the p75 TNF-R (the p75IC-binding proteins), and of the FAS-R (the FAS-IC-binding proteins). These p55IC-, p75IC- and FAS-IC- binding proteins may act as mediators or modulators of the TNF or FAS-R ligand effect on cells by way of mediating

or modulating the intracellular signaling process which usually occurs following the binding of TNF to the p55 and/or p75 TNF-R, or the binding of the FAS-R ligand at the cell surface. Further, it has been surprisingly and unexpectedly found that the p55IC and FAS-IC are capable of self association and that fragments of the p55IC and FAS-IC are similarly capable of binding to the p55 IC, particularly the so-called 'death domains (DD) within the ICs of these receptors, i.e. the p55DD and FAS-DD. Thus, p55 IC and FAS-IC and their fragments also represent proteins capable of binding to the p55IC and FAS-IC and hence may be modulators of the TNF or FAS-R ligand effect on cells.

Accordingly, the present invention provides a DNA sequence encoding a protein capable of binding to one or more of the intracellular domains of one or more receptors belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF) superfamily of receptors.

In particular, the present invention provides a DNA sequence selected from the group consisting of :

(a) a cDNA sequence derived from the coding region of a native TNF-R intracellular domain-binding protein;

(b) DNA sequences capable of hybridization to a DNA of (a) under moderately stringent conditions and which encode a biologically active TNF-R intracellular domain-binding protein; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active TNF-R intracellular domain-binding protein.

The present invention also provides a DNA sequence selected from the group consisting of :

(a) a cDNA sequence derived from the coding region of a native FAS-R intracellular domain-binding protein;

(b) DNA sequences capable of hybridization to a cDNA of (a) under moderately stringent conditions and which encode a biologically active FAS-R intracellular domain-binding protein; and

(c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active FAS-R intracellular domain-binding protein.

In embodiments of the present invention the DNA sequences encode p55 TNF-R, p75 TNF-R and FAS-R intracellular domain-binding proteins, such as those encoding the herein designated proteins 55.1, 55.3, 55.11, 75.3, 75.16, HF1, F2, F9 and DD11.

The present invention also provides a protein or analogs or derivatives thereof encoded by any of the above sequences of the invention, said proteins, analogs and derivatives being capable of binding to one or more of the intracellular domains of one or more TNF-Rs or FAS-R. Embodiments of this aspect of the invention include the herein designated proteins 55.1, 55.3, 55.11, 75.3, 75.16, HF1, F2, F9 and DD11, their analogs and their derivatives.

Also provided by the present invention are vectors encoding the above proteins of the invention, which contain the above DNA sequences of the invention, these vectors being capable of being expressed in suitable eukaryotic or prokaryotic host cells; transformed eukaryotic or prokaryotic host cells containing such vectors; and a method for producing the proteins, analogs or derivatives of the invention by growing such transformed host cells under conditions suitable for the expression of said protein, analogs or derivatives, effecting post-translational modifications of said protein as necessary for obtention of said protein and extracting said expressed protein, analogs or derivatives from the culture medium of said transformed cells or from cell extracts of said transformed cells.

In another aspect, the present invention also provides antibodies or active derivatives or fragments thereof specific to the proteins, analogs and derivatives thereof, of the invention.

By yet another aspect of the invention, there are provided various uses of the above DNA sequences or the proteins which they encode, according to the invention, which uses include amongst others :

(i) a method for the modulation of the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R, comprising treating said cells with one or more

proteins, analogs or derivatives selected from the group consisting of the proteins, analogs and derivatives, according to the invention, and a protein being the p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives thereof, all of said proteins being capable of binding to the intracellular domain and modulating the activity of said TNF-R or FAS-R, wherein said treating of the cells comprises introducing into said cells said one or more proteins, analogs or derivatives in a form suitable for intracellular administration or introducing into said cells, in the form of a suitable expression vector, the DNA sequence encoding said one or more proteins, analogs or derivatives ;

(ii) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R comprising treating said cells with antibodies or active derivatives or fragments thereof according to the invention;

(iii) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or FAS-R comprising treating said cells with an oligonucleotide sequence encoding an antisense sequence of at least part of the sequence according to the invention, or encoding an antisense sequence of the p55IC, p55DD, FAS-IC, or FAS-DD sequence, said oligonucleotide sequence being capable of blocking the expression of at least one of the TNF-R or FAS-R intracellular domain binding proteins;

(iv) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or FAS-R comprising :

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein that is capable of binding to a specific cell surface receptor and a sequence selected from an oligonucleotide sequence encoding an antisense sequence of at least part of the sequence according to the invention and an oligonucleotide sequence encoding an antisense sequence of the p55IC, p55DD, FAS-IC, or FAS-DD sequence, said oligonucleotide sequence being capable of blocking the expression of at least one of the TNF-R or FAS-R intracellular domain binding proteins when introduced into said cells by said virus ; and

(b) infecting said cells with said vector of (a).

(v) a method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R, comprising treating said cells with a suitable vector encoding a ribozyme having a sequence specific to a sequence selected from an mRNA sequence encoding a protein, analog or derivative of the invention and an mRNA sequence encoding the p55IC, p55DD, FAS-IC or FAS-DD, said ribozyme sequence capable of interacting with said mRNA sequence and capable of cleaving said mRNA sequence resulting in the inhibition of the expression of the protein, analog or derivative of the invention or of the expression of the p55IC, p55DD, FAS-IC or FAS-DD.

(vi) a method for treating tumor cells or HIV-infected cells comprising:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein that is capable of binding to a tumor cell surface receptor or HIV-infected cell surface receptor and a sequence selected from a sequence according to the invention encoding a protein, analog or derivative of the invention and a sequence encoding the p55IC, p55DD, FAS-IC, FAS-DD, or a biologically active analog or derivative thereof, said protein, analog or derivative of the invention, p55IC, p55DD, FAS-IC, FAS-DD, analog or derivative, when expressed in said tumor cell or HIV-infected cell being capable of killing said cell; and

(b) infecting said tumor cells or HIV-infected cells with said vector of (a).

(vii) a method for isolating and identifying proteins, factors or receptors capable of binding to the intracellular domain binding proteins according to the invention, comprising applying the procedure of affinity chromatography in which said protein according to the invention is attached to the affinity chromatography matrix, said attached protein is brought into contact with a cell extract and proteins, factors or receptors from cell extract which bound to said attached protein are then eluted, isolated analyzed;

(viii) a method for isolating and identifying proteins, capable of binding to the intracellular domain binding proteins according to the invention, comprising applying the yeast two-hybrid procedure in which a sequence encoding said intracellular domain binding protein is carried by one hybrid vector and a sequence from a cDNA or genomic DNA library is carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said intracellular domain binding protein; and

(ix) a method for isolating and identifying a protein capable of binding to the intracellular domains of TNF-Rs or FAS-R comprising applying the procedure of non-stringent southern hybridization followed by PCR cloning, in which a sequence or parts thereof according to the invention is used as a probe to bind sequences from a cDNA or genomic DNA library, having at least partial homology thereto, said bound sequences then amplified and cloned by the PCR procedure to yield clones encoding proteins having at least partial homology to said sequences according to the invention.

The present invention also provides a pharmaceutical composition for the modulation of the TNF- or FAS ligand- effect on cells comprising, as active ingredient, any one of the following : (i) a protein according to the invention, or the protein p55IC, p55DD, FAS-IC or FAS-DD, its biologically active fragments, analogs, derivatives or mixtures thereof; (ii) a recombinant animal virus vector encoding a viral surface protein capable of binding to a TNF-R or FAS-R - carrying cell - or tumor cell-specific receptor and a sequence encoding a protein, analog or derivative of the invention or encoding the p55IC, p55DD, FAS-IC or FAS-DD; (iii) a recombinant animal virus vector encoding a viral surface protein as in (ii) above and an oligonucleotide sequence encoding an antisense sequence of the p55IC, p55DD, FAS-IC or FAS-DD sequence; and (iv) a vector encoding a ribozyme of sequence capable of interacting with a mRNA sequence encoding a protein,

analog or derivative of the invention or a mRNA sequence encoding the p55IC, p55DD, FAS-IC or FAS-DD.

"Modulation of the TNF-effect on cells" is understood to encompass *in-vitro* as well as *in-vivo* treatment.

Brief Description of the Drawings

Figs 1a-c depict schematically the partial and preliminary nucleotide sequence of cDNA clones encoding the p55IC and p75IC-binding proteins, wherein Fig. 1(a) is the partial and preliminary sequence of clone 55.11 encoding the p55IC-binding protein 55.11; Fig. 1(b) is the partial and preliminary sequence of clone 75.3 encoding the p75IC-binding protein 75.3; and Fig. 1(c) is the partial and preliminary sequence of clone 75.16 encoding the p75IC-binding protein p75.16; all as described in Example 1.

Fig. 2 is a reproduction of a Western blot stained with anti-MBP polyclonal antiserum, showing the self association of the p55IC, the Western blot derived from an SDS-PAGE gel on which were electrophoresed the interacting bacterially-produced chimeric proteins p55IC-MBP and p55IC-GST (lanes 1-4) or the control interaction between the chimeric protein p55IC-MBP and GST alone (lanes 5-8), the interactions between the chimeric proteins (and control) being carried out on glutathion-agarose beads prior to SDS-PAGE, as described in Example 2.

Fig. 3 is a reproduction of phase contrast micrographs showing the cytotoxic effect of the full-length p55IC in HTta1 cells transfected with an expression vector encoding this p55IC (right panel); and the inhibition of this cytotoxic effect when expression of the vector is blocked by treating the cells with tetracycline (left panel), as described in Example 2.

Fig. 4 (A and B) depicts graphically the ligand independent triggering of a cytocidal effect in HeLa cells transfected with p55R or parts thereof, or with FAS-IC, wherein in Fig. 4A there is depicted the results with respect to the p55R or parts thereof and in Fig. 4B there is depicted the results with respect to the FAS-IC. In the left hand

panels of both Fig. 4A and B there is depicted schematically the portion of the p55R or FAS-IC used in the transfections while the right hand panels depict graphically the experimental results, all as described in Example 2.

Fig. 5 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called 'HF1', which encodes a protein capable of binding to the intracellular domain of FAS-R (FAS-IC) and a region within the FAS-IC, the FAS-R 'death domain' (FAS-DD), as described in Example 3.

Fig. 6 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called 'F2', which encodes a protein capable of binding to the p55IC and FAS-IC, as described in Example 3.

Fig. 7 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called F9, which encodes a protein capable of binding to the p55IC and FAS-IC, as described in Example 3.

Fig. 8 depicts schematically the partial and preliminary nucleotide sequence of a cDNA clone, called DD11, which encodes a protein capable of binding to the p55IC, especially the p55DD, and FAS-IC, as described in Example 3.

Detailed Description of the Invention

The present invention relates, in one aspect, to novel proteins which are capable of binding to the intracellular domain of receptors belonging to the TNF/NGF superfamily, such as TNF-Rs and FAS-R and hence are considered as mediators or modulators of this superfamily of receptors, e.g. of the TNF-Rs and FAS-R, having a role in, for example, the signaling process that is initiated by the binding of TNF to the TNF-R and FAS ligand to FAS-R. Examples of these proteins are those which bind to the intracellular domain of the p55 TNF-R (p55IC), such as the proteins designated herein as 55.1, 55.3 and 55.11 (Example 1) as well as those encoded by cDNA clones F2, F9, and DD11 (Example 3); those which bind to the intracellular domain of the p75 TNF-R (p75IC), such as the proteins designated herein as 75.3 and 75.16 (Example 1); and those which bind to the intracellular domain of FAS-R (FAS-IC), such as the proteins encoded by cDNA clones HF1, F2, F9 and DD11 (Example 3). Proteins 55.1 and 55.3 have been found to

represent portions or fragments of the intracellular domain of the p55 TNF-R (p55IC); other proteins, 55.11, 75.3 and 75.16, represent proteins not described at all prior to filing of IL 109632 (75.3, 75.16) or those that have been described (55.11, see Khan et al., 1992) but whose function and other characteristics, particularly, the ability to bind to a TNF-R, were not described in any way (see Example 1, below). The new proteins encoded by cDNA clones HF1, F2, F9 and DD11 also represent proteins previously not described at all, i.e. their sequence is not in the 'GENEBANK' or 'PROTEIN BANK' data banks of DNA or amino acid sequences.

Thus, the present invention concerns the DNA sequences encoding these proteins and the proteins encoded by these sequences.

Moreover, the present invention also concerns the DNA sequences encoding biologically active analogs and derivatives of these proteins, and the analogs and derivatives encoded thereby. The preparation of such analogs and derivatives is by standard procedure (see for example, Sambrook et al., 1989) in which in the DNA sequences encoding these proteins, one or more codons may be deleted, added or substituted by another, to yield analogs having a least a one amino acid residue change with respect to the native protein. Acceptable analogs are those which retain at least the capability of binding to the intracellular domain of the TNF/NGF receptor superfamily, such as FAS-R or TNF-R, e.g. the p55IC, p75IC or FAS-IC, or which can mediate any other binding or enzymatic activity, e.g. analogs which bind the p55, p75IC or FAS-IC but which do not signal, i.e. do not bind to a further downstream receptor, protein or other factor, or do not catalyze a signal-dependent reaction. In such a way analogs can be produced which have a so-called dominant-negative effect, namely, an analog which is defective either in binding to the, for example, p55IC, p75IC or FAS-IC, or in subsequent signaling following such binding. Such analogs can be used, for example, to inhibit the TNF effect by competing with the natural IC-binding proteins. Likewise, so-called dominant-positive analogs may be produced which would serve to enhance, for example, the TNF or FAS ligand effect. These would have the same or better IC-binding properties and the same or better signaling properties of the natural IC-binding proteins. Similarly,

derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of the proteins, or by conjugation of the proteins to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art.

The new TNF-R and FAS-R intracellular domain - binding proteins, e.g. the proteins 55.1, 55.3, 55.11, 75.3, 75.16 as well as the proteins encoded by cDNA clones HF1, F2, F9 and DD11 (hereinafter, HF1, F2, F9 and DD11) have a number of possible uses, for example:

(i) They may be used to mimic or enhance the function of TNF or FAS-R ligand, in situations where an enhanced TNF or FAS-R ligand effect is desired such as in anti-tumor, anti-inflammatory or anti-HIV applications where the TNF-or FAS-R ligand- induced cytotoxicity is desired. In this case the proteins, e.g. those binding to the p55IC such as 5.1, 55.3, as well as F2, F9 and DD11, and the free p55IC itself (see below and Example 2), as well as the 'death domain' of the p55IC (p55DD - see IL 111125), which enhance the TNF effect; or proteins HF1, F2, F9 and DD11 as well as FAS-IC and FAS-DD which enhance the FAS-R ligand effect, i.e. cytotoxic effect, may be introduced to the cells by standard procedures known per se. For example, as the proteins are intracellular and it is desired that they be introduced only into the cells where the TNF or FAS-R ligand effect is wanted, a system for specific introduction of these proteins into the cells is necessary. One way of doing this is by creating a recombinant animal virus e.g. one derived from Vaccinia, to the DNA of which the following two genes will be introduced: the gene encoding a ligand that binds to cell surface proteins specifically expressed by the cells e.g. ones such as the AIDs (HIV) virus gp120 protein which binds specifically to some cells (CD4 lymphocytes and related leukemias) or any other ligand that binds specifically to cells carrying a TNF-R or FAS-R, such that the recombinant virus vector will be capable of binding such TNF-R- or FAS-R-carrying cells; and the gene encoding the new intracellular domain-binding protein or the p55IC, p55DD, FAS-IC or FAS-DD protein. Thus, expression of the cell-surface-binding protein on the surface of the virus will target the virus specifically

to the tumor cell or other TNF-R- or FAS-R- carrying cell, following which the intracellular domain-binding protein encoding sequence or p55IC, p55DD, FAS-IC or FAS-DD encoding sequence will be introduced into the cells via the virus, and once expressed in the cells will result in enhancement of the TNF or FAS-R ligand effect leading to the death of the tumor cells or other TNF-R- or FAS-R- carrying cells it is desired to kill. Construction of such recombinant animal virus is by standard procedures (see for example, Sambrook et al., 1989). Another possibility is to introduce the sequences of the new proteins or the p55IC, p55DD, FAS-IC or FAS-DD in the form of oligonucleotides which can be absorbed by the cells and expressed therein.

(ii) They may be used to inhibit the TNF or FAS-R ligand effect, e.g. in cases such as tissue damage in septic shock, graft-vs.-host rejection, or acute hepatitis, in which case it is desired to block the TNF-induced TNF-R or FAS-R ligand induced FAS-R intracellular signaling. In this situation it is possible, for example, to introduce into the cells, by standard procedures, oligonucleotides having the anti-sense coding sequence for these new proteins, or the anti-sense coding sequence for p55IC, p55DD, FAS-IC or FAS-DD, which would effectively block the translation of mRNAs encoding these proteins and thereby block their expression and lead to the inhibition of the TNF- or FAS-R ligand- effect.

Such oligonucleotides may be introduced into the cells using the above recombinant virus approach, the second sequence carried by the virus being the oligonucleotide sequence. Another possibility is to use antibodies specific for these proteins to inhibit their intracellular signaling activity. It is possible that these new proteins have an extracellular domain as well as an intracellular one, the latter which binds to the TNF-R or FAS-R binding domain, and thus antibodies generated to their extracellular domains can be used to block their TNF- or FAS-R ligand- related functions.

Yet another way of inhibiting the TNF or FAS-R ligand effect is by the recently developed ribozyme approach. Ribozymes are catalytic RNA molecules

that specifically cleave RNAs. Ribozymes may be engineered to cleave target RNAs of choice, e.g. the mRNAs encoding the new proteins of the invention or the mRNA encoding the p55IC, p55DD, FAS-IC or FAS-DD. Such ribozymes would have a sequence specific for the mRNA of choice and would be capable of interacting therewith (complementary binding) followed by cleavage of the mRNA, resulting in a decrease (or complete loss) in the expression of the protein it is desired to inhibit, the level of decreased expression being dependent upon the level of ribozyme expression in the target cell. To introduce ribozymes into the cells of choice (e.g. those carrying TNF-Rs or FAS-R) any suitable vector may be used, e.g. plasmid, animal virus (retrovirus) vectors, that are usually used for this purpose (see also (i) above, where the virus has, as second sequence, a cDNA encoding the ribozyme sequence of choice). Moreover, ribozymes can be constructed which have multiple targets (multi-target ribozymes) that can be used, for example, to inhibit the expression of one or more of the proteins of the invention and/or the p55IC, p55DD, FAS-IC or FAS-DD as well (For reviews, methods etc. concerning ribozymes see Chen et al., 1992; Zhao and Pick, 1993; Shore et al., 1993; Joseph and Burke, 1993; Shimayama et al., 1993; Cantor et al., 1993; Barinaga, 1993; Crisell et al., 1993 and Koizumi et al., 1993).

(iii) They may be used to isolate, identify and clone other proteins which are capable of binding to them, e.g. other proteins involved in the intracellular signaling process that are downstream of the TNF-R or FAS-R intracellular domain. In this situation, these options, namely, the DNA sequences encoding them may be used in the yeast two-hybrid system (see Example 1, below) in which the sequence of these proteins will be used as "baits" to isolate, clone and identify from cDNA or genomic DNA libraries other sequences ("preys") encoding proteins which can bind to these new TNF-R or FAS-R intracellular domain-binding proteins. In the same way, it may also be determined whether the specific proteins of the present invention, namely, those which bind to the p55IC, p75IC, or FAS-IC, can bind to other receptors of the TNF/NGF superfamily of receptors. For example, it has

recently been reported (Schwalb et al., 1993; Baens et al., 1993; Crowe et al., 1994) that there exist other TNF-Rs besides the p55 and p75 TNF-Rs. Accordingly, using the yeast two-hybrid system it may be specifically tested whether the proteins of the present invention are capable of specifically binding to these other TNF-Rs or other receptors of the TNF/NGF superfamily. Moreover, this approach may also be taken to determine whether the proteins of the present invention are capable of binding to other known receptors in whose activity they may have a functional role.

(iv) The new proteins may also be used to isolate, identify and clone other proteins of the same class i.e. those binding to TNF-R or FAS-R intracellular domains or to functionally related receptors, and involved in the intracellular signaling process. In this application the above noted yeast two-hybrid system may be used, or there may be used a recently developed (Wilks et al., 1989) system employing non-stringent southern hybridization followed by PCR cloning. In the Wilks et al. publication, there is described the identification and cloning of two putative protein-tyrosine kinases by application of non-stringent southern hybridization followed by cloning by PCR based on the known sequence of the kinase motif, a conceived kinase sequence. This approach may be used, in accordance with the present invention using the sequences of the new proteins to identify and clone those of related TNF-R, FAS-R or related receptor (TNF/NGF superfamily receptors) intracellular domain-binding proteins.

(v) Yet another approach to utilizing the new proteins of the invention is to use them in methods of affinity chromatography to isolate and identify other proteins or factors to which they are capable of binding, e.g. other receptors related to TNF-Rs (TNF/NGF receptor superfamily) or other proteins or factors involved in the intracellular signaling process. In this application, the proteins of the present invention, may be individually attached to affinity chromatography matrices and then brought into contact with cell extracts or isolated proteins or factors suspected of being involved in the intracellular signaling process. Following the

affinity chromatography procedure, the other proteins or factors which bind to the new proteins of the invention, can be eluted, isolated and characterized.

(vi) As noted above, the new proteins of the invention may also be used as immunogens (antigens) to produce specific antibodies thereto. These antibodies may also be used for the purposes of purification of the new proteins either from cell extracts or from transformed cell lines producing them. Further, these antibodies may be used for diagnostic purposes for identifying disorders related to abnormal functioning of the TNF or FAS-R ligand system, e.g. overactive or underactive TNF- or FAS-R ligand- induced cellular effects. Thus, should such disorders be related to a malfunctioning intracellular signaling system involving the new proteins, such antibodies would serve as an important diagnostic tool.

As regards such antibodies, the term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, anti-idiotypic (anti-Id) antibodies to antibodies that can be labeled in soluble or bound form, as well as fragments thereof provided by any known technique, such as, but not limited to enzymatic cleavage, peptide synthesis or recombinant techniques.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen. A monoclonal antibody contains a substantially homogeneous population of antibodies specific to antigens, which populations contains substantially similar epitope binding sites. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, Nature, 256:495-497 (1975); U.S. Patent No. 4,376,110; Ausubel et al., eds., Harlow and Lane ANTIBODIES : A LABORATORY MANUAL, Cold Spring Harbor Laboratory (1988); and Colligan et al., eds., Current Protocols in Immunology, Greene publishing Assoc. and Wiley Interscience N.Y., (1992, 1993), the contents of which references are incorporated entirely herein by reference. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, GILD and any subclass thereof. A hybridoma producing a mAb of the present invention may be cultivated *in vitro, in situ* or *in vivo*. Production of high titers of mAbs *in vivo* or *in situ* makes this the presently preferred method of production.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having the variable region derived from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies are primarily used to reduce immunogenicity in application and to increase yields in production, for example, whre murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the art (Cabilly et al., *Proc. Natl. Acad. Sci. USA* 81:3273-3277 (1984); Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne et al., *Nature* 312:643-646 (1984); Cabilly et al., European Patent Application 125023 (published November 14, 1984); Neuberger et al., *Nature* 314:268-270 (1985); Taniguchi et al., European Patent Application 171496 (published February 19, 1985); Morrison et al., European Patent Application 173494 (published March 5, 1986); Neuberger et al., PCT Application WO 8601533, (published March 13, 1986); Kudo et al., European Patent Application 184187 (published June 11, 1986); Sahagan et al., *J. Immunol.* 137:1066-1074 (1986); Robinson et al., International Patent Application No. WO8702671 (published May 7, 1987); Liu et al., *Proc. Natl. Acad. Sci USA* 84:3439-3443 (1987); Sun et al., *Proc. Natl. Acad. Sci USA* 84:214-218 (1987); Better et al., *Science* 240:1041-1043 (1988); and Harlow and Lane, ANTIBODIES :A LABORATORY MANUAL, supra. These references are entirely incorporated herein by reference.

An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Patent No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the IC-binding proteins, analogs or derivatives thereof, of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD, analogs or derivatives thereof may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for an epitope of the above IC-binding proteins, analogs or derivatives or p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as GRB protein-α.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

It will be appreciated that Fab and $F(ab')_2$ and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the IC-binding proteins or p55IC, p55DD, FAS-IC or FAS-DD according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The

term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the IC-binding proteins or p55IC, p55DD, FAS-IC, FAS-DD in a sample or to detect presence of cells which express the IC-binding proteins of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD proteins. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of IC-binding proteins of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD. *In situ* detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the IC-binding proteins or the p55IC, p55DD, FAS-IC, FAS-DD, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide

variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for IC-binding proteins of the present invention or the p55IC, p55DD, FAS-IC, FAS-DD, typically comprises incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capably of identifying the IC-binding proteins or the p55IC, p55DD, FAS-IC, FAS-DD, and detecting the antibody by any of a number of techniques well known in the art.

The biological sample may be treated with a solid phase support or carrier such as nitrocellulose, or other solid support or carrier which is capable of immobilizing cells, cell particles or soluble proteins. The support or carrier may then be washed with suitable buffers followed by treatment with a detectably labeled antibody in accordance with the present invention, as noted above. The solid phase support or carrier may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support or carrier may then be detected by conventional means.

By "solid phase support", "solid phase carrier", "solid support", "solid carrier", "support" or "carrier" is intended any support or carrier capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support or carrier configuration may be spherical, as in a bead, cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports or carriers include polystyrene beads. Those skilled in the art will know may other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of antibody, of the invention as noted above, may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which an antibody in accordance with the present invention can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomeras, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactivity labeling the antibodies or antibody fragments, it is possible to detect R-PTPase through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S. et al., North Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

It is also possible to label an antibody in accordance with the present invention with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be then detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrine, pycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}E, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriamine pentaacetic acid (ETPA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

An antibody molecule of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support or carrier and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support or carrier is washed to

remove the residue of the fluid sample, including unreacted antigen, if any, and the contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support or carrier through the unlabeled antibody, the solid support or carrier is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support or carrier and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support or carrier is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support or carrier is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support or carrier after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support or carrier is then determined as in the "simultaneous" and "forward" assays.

The new proteins of the invention may be produced by any standard recombinant DNA procedure (see for example, Sambrook, et al., 1989) in which suitable eukaryotic or prokaryotic host cells are transformed by appropriate eukaryotic or prokaryotic vectors containing the sequences encoding for the proteins. Accordingly, the present invention also concerns such expression vectors and transformed hosts for the production of the proteins of the invention. As mentioned above, these proteins also include their biologically active analogs and derivatives, and thus the vectors encoding them also include vectors encoding analogs of these proteins, and the transformed hosts include those producing such analogs.

The derivatives of these proteins are the derivatives produced by standard modification of the proteins or their analogs, produced by the transformed hosts.

In another aspect, the invention relates to the use of the free intracellular domain of the p55 TNR-R (p55IC) or FAS-R (FAS-IC) or their so-called 'death domains' (p55DD or FAS-DD, respectively) as an agent for enhancing the TNF or FAS-R ligand effect on cells, on its own (see Example 2). Where it is desired to introduce a TNF- or FAS-R-ligand-induced cytotoxic effect in cells, e.g. cancer cells or HIV-infected cells, the p55IC, p55DD, FAS-IC or FAS-DD can be introduced into such cells using the above noted (see (i) above) recombinant animal virus (e.g. vaccinia) approach. Here too, the native p55IC, p55DD, FAS-IC or FAS-DD, biologically active analogs and derivatives or fragments may be used, all of which can be prepared as noted above.

Likewise, the present invention also relates to the specific blocking of the TNF-effect or FAS-R ligand-effect by blocking the activity of the p55IC, p55DD, FAS-IC or FAS-DD, e.g. anti-sense oligonucleotides may be introduced into the cells to block the expression of the p55IC, p55DD, FAS-IC or FAS-DD.

The present invention also relates to pharmaceutical compositions comprising recombinant animal virus vectors encoding the TNF-R or FAS-R intracellular domain binding proteins (including the p55IC, p55DD, FAS-IC and FAS-DD), which vector also encodes a virus surface protein capable of binding specific target cell (e.g. cancer cells) surface proteins to direct the insertion of the intracellular domain binding protein sequences into the cells. Other aspects of the invention will be apparent from the following examples.

The invention will now be described in more detail in the following non-limiting examples and the accompanying drawings :

EXAMPLE 1

Cloning and isolation of proteins which bind to the intracellular domains of the p55 and p75 TNF receptors

This example is set forth also in IL 109632, and is included herein because of its detailed description of the method for isolating and identifying other TNF/NGF receptor

superfamily intracellular domain-binding proteins, e.g. those set forth in Example 3 below. It should be noted, however, that some additions have been made in this example to incorporate new experimental data, e.g. an update of the hereinbelow designated protein 55.11 (see Fig. 1 (a)).

To isolate proteins interacting with the intracellular domains of the p55 and p75 TNF receptors (p55IC and p75 IC), the yeast two-hybrid system was used (Fields and Song, 1989). Briefly, this two-hybrid system is a yeast-based genetic assay to detect specific protein-protein interactions *in vivo* by restoration of a eukaryotic transcriptional activator such as GAL4 that has two separate domains, a DNA binding and an activation domain, which domains when expressed and bound together to form a restored GAL4 protein, is capable of binding to an upstream activating sequence which in turn activates a promoter that controls the expression of a reporter gene, such as lacZ or HIS3, the expression of which is readily observed in the cultured cells. In this system the genes for the candidate interacting proteins are cloned into separate expression vectors. In one expression vector the sequence of the one candidate protein is cloned in phase with the sequence of the GAL4 DNA-binding domain to generate a hybrid protein with the GAL4 DNA-binding domain, and in the other vector the sequence of the second candidate protein is cloned in phase with the sequence of the GAL4 activation domain to generate a hybrid protein with the GAL4-activation domain. The two hybrid vectors are then co-transformed into a yeast host strain having a lacZ or HIS3 reporter gene under the control of upstream GAL4 binding sites. Only those transformed host cells (cotransformants) in which the two hybrid proteins are expressed and are capable of interacting with each other, will be capable of expression of the reporter gene. In the case of the lacZ reporter gene, host cells expressing this gene will become blue in color when X-gal is added to the cultures. Hence, blue colonies are indicative of the fact that the two cloned candidate proteins are capable of interacting with each other.

Using this two-hybrid system, the intracellular domains p55IC and p75IC were cloned, separately, into the vector pGBT9 (carrying the GAL4 DNA-binding sequence, provided by CLONTECH, USA, see below), to create fusion proteins with the GAL4

DNA-binding domain (similarly, the intracellular domain, FAS-IC and a portion of the 55IC, namely, the 55DD were also cloned into pGBT9 and used to isolate other IC-binding proteins, see Example 3 below). For the cloning of p55IC and p75IC into pGBT9, clones encoding the full-length cDNA sequences of p55 TNF-R (Schall et al., 1990) and p75 TNF-R (Smith et al., 1990) were used from which the intracellular domains (IC) were excised as follows : p55IC was excised using the enzymes EcoRI and SalI, the EcoRI-SalI fragment containing the p55IC sequence was then isolated by standard procedures and inserted into the pGBT9 vector opened, in its multiple cloning site region (MCS), with EcoRI and SalI. p75 IC was excised using the enzymes BspHI and SalI, the BspHI-SalI fragment containing the p75 IC sequence was then isolated by standard procedures and filled-in with the Klenow enzyme to generate a fragment which could be inserted into the pGBT9 vector opened with SmaI and SalI.

The above hybrid (chimeric) vectors were then cotransfected (separately, one cotransfection with the p55IC hybrid and one with the p75 IC hybrid vector) together with a cDNA library from human HeLa cells cloned into the pGAD GH vector, bearing the GAL4 activating domain, into the HF7c yeast host strain (all the above-noted vectors, pGBT9 and pGAD GH carrying the HeLa cell cDNA library, and the yeast strain were purchased from Clontech Laboratories, Inc., USA, as a part of MATCHMAKER Two-Hybrid System, #PT1265-1). The co-transfected yeasts were selected for their ability to grow in medium lacking Histidine (His^- medium), growing colonies being indicative of positive transformants. The selected yeast clones were then tested for their ability to express the lacZ gene, i.e. for their LAC Z activity, and this by adding X-gal to the culture medium, which is catabolized to form a blue colored product by β-galactosidase, the enzyme encoded by the lacZ gene. Thus, blue colonies are indicative of an active lacZ gene. For activity of the lacZ gene, it is necessary that the GAL4 transcription activator be present in an active form in the transformed clones, namely that the GAL4 DNA-binding domain encoded by one of the above hybrid vectors be combined properly with the GAL4 activation domain encoded by the other hybrid vector. Such a combination is only possible if the two proteins fused to each of the GAL4 domains are capable of stably interacting

(binding) to each other. Thus, the His^+ and blue (LAC Z^+) colonies that were isolated are colonies which have been cotransfected with a vector encoding p55IC and a vector encoding a protein product of human HeLa cell origin that is capable of binding stably to p55 IC; or which have been transfected with a vector encoding p75IC and a vector encoding a protein product of human HeLa cell origin that is capable of binding stably to p75 IC.

The plasmid DNA from the above His^+, LAC Z^+ yeast colonies was isolated and electroporated into E. coli strainHB101 by standard procedures followed by selection of Leu^+ and Ampicillin resistant transformants, these transformants being the ones carrying the hybrid pGAD GH vector which has both the Amp^R and Leu^2 coding sequences. Such transformants therefore are clones carrying the sequences encoding newly identified proteins capable of binding to the p55IC or p75IC. Plasmid DNA was then isolated from these transformed E. coli and retested by :

(a) retransforming them with the original intracellular domain hybrid plasmids (hybrid pGTB9 carrying either the p55IC or p75IC sequences) into yeast strain HF7 as set forth hereinabove. As controls, vectors carrying irrelevant protein encoding sequences, e.g. pACT-lamin or pGBT9 alone were used for cotransformation with the p55IC-binding protein or p75IC-binding protein encoding plasmids. The cotransformed yeasts were then tested for growth on His^- medium alone, or with different levels of 3-aminotriazole; and

(b) retransforming the plasmid DNA and original intracellular domain hybrid plasmids and control plasmids described in (a) into yeast host cells of strain SFY526 and determining the LAC Z^+ activity (effectivity of β-gal formation, i.e. blue color formation).

The results of the above tests revealed that the pattern of growth of colonies in His^- medium was identical to the pattern of LAC Z activity, as assessed by the color of the colony, i.e. His^+ colonies were also LAC Z^+. Further, the LAC Z activity in liquid culture (preferred culture conditions) was assessed after transfection of the GAL4 DNA-binding and activation-domain hybrids into the SFY526 yeast hosts which have a better LAC Z inducibility with the GAL4 transcription activator than that of the HF-7 yeast host cells.

The results of the above co-transfections are set forth in Table 1 below, from which it is apparent that a number of proteins were found that were capable of binding to the p55IC or the p75IC, namely, the proteins designated 55.11, which binds to the p55IC; and 75.3 and 75.16 which bind to the p75IC. All of these p55IC- and p75IC-binding proteins are authentic human proteins all encoded by cDNA sequences originating from the HeLa cell cDNA library, which were fused to the GAL4 activation-domain sequence in the plasmid pGAD GH in the above yeast two-hybrid analysis system.

Interestingly, it was also found that fragments of the p55IC, itself, namely, the proteins designated 55.1 and 55.3 were capable of binding to p55IC. These are discussed also in Example 2 below.

TABLE 1

SUMMARY OF THE CHARACTERISTICS OF SOME OF THE cDNA CLONES (SEE ALSO EXAMPLE 3) ISOLATED BY THE TWO-HYBRIDSYSTEM APPROACH

DNA-binding domain hybrid	Activation-domain hybrid	Colony color	Lac Z activity in liquid culture assay
pGBT9-IC55	---	white	0.00
pGBT9-IC55	55.1	blue	0.65
pGBT9-IC55	55.3	blue	0.04
---	55.1	white	0.00
---	55.3	white	0.00
pACT-Lamin	55.1	white	0.00
pACT-Lamin	55.3	white	0.00
pGBT9	55.1	white	0.00
pGBT9	55.3	white	0.00
pGBT9-IC55	55.11	blue	ND
---	55.11	white	ND
pACT-Lamin	55.11	white	ND
pGBT9	55.11	white	ND
pGBT9-IC75	75.3	blue	ND
pGBT9-IC75	---	white	ND
---	75.3	white	ND
pACT-Lamin	75.3	white	ND
pGBT9	75.3	white	ND
pGBT9-IC75	75.16	blue	ND
---	75.16	white	ND
pACT-Lamin	75.16	white	ND
pGBT9	75.16	white	ND

In the above Table 1, the plasmids and hybrid encoding the GAL4 DNA-binding domain and GAL4 activation domain are as follows :

DNA-binding domain hybrids :

pGBT9-IC55 : full-length intracellular domain of the p55-TNF-R (p55IC)

pACT-Lamin : irrelevant protein - lamin.

pGBT9 : vector alone

pGBT9-IC75 : full-length intracellular domain of the p75-TNF-R (p75IC)

Activation-domain hybrid :

55.1 and 55.3 correspond to fragments of the intracellular domain of the p55-TNF-R.

55.11 : is the novel protein associating with the p55-TNF-R

75.3 and 75.16 are the novel proteins associating with the p75-TNF-R.

The above noted cloned cDNAs encoding the novel p55IC- and p75IC- binding proteins, 55.11, 75.3 and 75.16, were then sequenced using standard DNA sequencing procedures. The partial sequence of all of these protein-encoding sequences is set forth in Figs. 1 a-c, where Fig. 1(a) depicts an updated version (i.e. post-IL109632) of the partial sequence of the cDNA encoding protein 55.11; Fig. 1(b) depicts the partial sequence of the cDNA encoding protein 75.3; and Fig. 1(c) depicts the partial sequence of the cDNA encoding protein 75.16.

It should be noted, however, that the partial sequence of the cDNA encoding the 55.11 protein has also been reported by Khan et al. (1992), in a study of human brain cDNA sequences, which study was directed at the establishment of a new rapid and accurate method for the sequencing and physical and genetic mapping of human brain cDNAs. However, Khan et al. did not provide any information as regards the function or any other characteristics of the protein encoded by the 55.11 cDNA sequence, such functional or other analysis not being the intention of Khan et al. in their study.

The above cDNA encoding protein 55.11 was then subjected to Northern blot analysis, in which the 55.11 cDNA was shown to hybridize to a mRNA transcript having a size of about 3kb, expressed in the following cell cultures: HeLa, Alexander (liver) and CEM (T cells).

EXAMPLE 2

Self-association ability of the intracellular domain of the p55 TNF receptor (p55IC) and its capability to cause cell death

This example is also from IL 109632 and is maintained herein because of its description of the self-associating capability of the p55IC and the ability of the p55IC to induce, in a ligand-independent fashion, cell cytotoxic effects. It should however be noted that some additions have been made to this example in view of new experimental data.

As set forth in Example 1 above, it was discovered that the intracellular domain of p55 TNF-R (p55IC) is capable of binding to itself, and further that fragments of p55IC, namely proteins 55.1 and 55.3, are also capable of binding to p55IC.

It is known that the binding of TNF to p55 TNF-R leads to a cytocidal effect on the cells carrying this receptor. Further, antibodies against the extracellular domain of this receptor can themselves trigger this effect, in correlation with the effectivity of receptor cross-linking by them.

In addition, mutational studies (Tartaglia et al., (1993); Brakebusch et al., (1992)) showed that the function of the p55-R depends on the integrity of its intracellular domain. It was therefore suggested that the initiation of signaling for the cytocidal effect of TNF occurs as a consequence of association of two or more intracellular domains of the p55-R (p55-IC), imposed by receptor aggregation. The results in accordance with the present invention provide further evidence for this notion, showing that expression of the intracellular domain of the p55-R within cells, without the transmembrane or intracellular domain, triggers their death. Such free intracellular domains of the p55-R are shown to self associate, which probably accounts for their ability to function independently of TNF. The fact that the signaling by the full length p55-R does depend on TNF stimulation is suggested to reflect activiti(es) of the transmembrane or extracellular domain of the receptor which decrease or prevent this self association.

The ability of the intracellular domain of the p55-R (p55-IC) to self associate was found serendipitously, in the attempts to clone effector proteins which interact with this receptor (see Example 1 above). We applied for that purpose the above mentioned "two

hybrid" technique. In addition to the novel protein, 55.11 found to associate (bind) to the p55IC, it was also found that three other cloned HeLa cell cDNAs contained cDNA sequences encoding for parts of the intracellular domain of the p55-R, implying that the p55-IC is capable of self-association. Two of these clones were identical, containing an insert which encodes for amino acids 328-426 (designated as clone 55.1 encoding protein fragment 55.1 of the p55IC). The third contained a longer insert, encoding for amino acids 277-426 (designated as clone 55.3 encoding protein fragment 55.3 of the p55IC).

In addition, we assessed the in vitro interaction between two bacterially produced chimeras of the p55IC, one, in which it was fused to the maltose binding protein (MBP) and the other in which is was fused to the glutathione-S-transferase (GST). These chimeras were constructed, cloned and expressed by standard methods. Following their expression, the assessment of the self-interaction of the p55-R intracellular domain (p55IC) by determining the interaction of the above bacterially-produced chimeric proteins GST-IC55 (Mr - 51kD) and MBP-IC55 (Mr - 67 kD) with each other. Equal amounts of the GST-IC55 chimera (samples of lanes 1-4 in Fig. 2) or GST alone (samples of lanes 5-8 in Fig. 2) were bound to glutathion-agarose beads (Sigma) and were then incubated with the same amount of MBP-IC55 fusion protein in one of the following buffer solutions :

(i) buffer I (20mM Tris-HCl, pH 7.5, 100mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 5mM DTT, 0.2% Triton X100, 0.5mM PMSF, 5% Glycerol). This was done for the samples of Lanes 1 and 5 of Fig. 2.

(ii) buffer I containing 5mM EDTA instead of $MgCl_2$. This was done for the samples of Lanes 2 and 6 of Fig. 2.

(iii) buffer I containing 250mM instead of 100mM KCl. This was done for the samples of Lanes 3 and 7 of Fig. 2.

(iv) buffer I containing 400mM instead of 100mM KCl. This was done for the samples of Lanes 4 and 8 of Fig. 2.

After incubation with rotation for 2h at 4°C, the beads were washed with the same buffers and then boiled in SDS-PAGE buffer followed by electrophoresis by PAGE. The proteins on the gel were then Western blotted to a nitrocellulose membrane which was

then stained with polyclonal antiserum against MBP. A reproduction of this stained Western blot is shown in Fig. 2, the samples in lanes 1-8 being those noted above.

From Fig. 2 it is apparent that the p55IC-MBP chimera bind to the p55IC-GST chimeria (lanes 1-4) independently of divalent cations and even at a rather high salt concentration (0.4M KCl). Thus, it is concluded that the p55IC is able to avidly self-associate.

To evaluate the functional implications of the propensity of the p55-IC to self associate, we attempted to express the p55-IC within the cytoplasm of cells which are sensitive to the cytocidal effect of TNF. Considering the possibility that the p55-IC will turn to be cytotoxic, we chose to express it in an inducible manner, using the recently developed, tightly regulated tetracycline-controlled mammalian expression system (Gossen and Boujard, 1992). Expression of the p55-IC resulted in massive cell death (Fig. 3, right panel). The dying cells displayed cell surface blabbing as observed in the killing of the cells by TNF. Transfection of the p55-IC construct to the cells in the presence of tetracycline, which reportedly decreases the expression of pHD10-3 regulated constructs by as much as 10^5 fold, still resulted in some cell death, although significantly less than that observed in the absence of tetracycline (Fig 3, left panel). In contrast, cells transfected with a control construct, containing the lucipherase cDNA, showed no signs of death (results not shown).

The ability of the p55-IC to trigger cell death, when expressed without the transmembrane or extracellular domains of the receptor, provides further evidence for the involvement of this domain in signaling. Furthermore, it indicates that no other part of the receptor plays a direct role in such signaling.. Studies of the effects of mutations, including those mutations studied in the present invention, on the function of the p55-IC, indicated that the region extending between amino acid residues 326 and 407 is most critical for its function. This region shows marked resemblance to sequences within the intracellular domains of two other receptors, evolutionarily related to the p55 TNF-R - namely, the Fas receptor (Itoh et al., 1991; Oehm et al., 1992), which can also signal for cell death and CD40 -a receptor (Stamenkovic et al., 1989) which enhances cell growth; this sequence therefore seems to constitute a conserved motif which plays some kind of general role in

signaling. Since it does not resemble known motives characteristic of enzymatic activities, it seems plausible that it signals in indirect manner, i.e. possibly by serving as a docking site for signaling enzymes or for proteins which transmit stimulatory signals to them. The p55-IC, the Fas receptor and CD 40 can all be stimulated by antibodies against their extracellular domain. Their stimulation could be shown to correlate with the ability of the antibodies to cross-link the receptors. It therefore seems that the signaling is initiated as a consequence of interaction of two or more intracellular domains imposed by aggregation of the extracellular domains. Involvement of such interaction in the initiation of signaling of these receptors was also indicated by studies (Brakebusch et al., 1992) showing that expression of receptors made nonfunctional by mutation of their intracellular domain, had a "dominant negative" effect on the function of co-expressed normal receptors. Aggregation of the p55-R in response to TNF was suggested to occur in a passive manner, merely due to the fact that each of the TNF molecules, which occur as homotrimers, can bind two or three receptor molecules. However, the findings of the present invention suggest that this process occurs somewhat differently.

The propensity of the p55-IC to self associate indicates that this domain plays an active role in its induced aggregation. Moreover, this activity of the p55-IC seems to suffice for initiating its signaling, since when expressed independently of the rest of the receptor molecule, it can trigger cell death in the absence of TNF or any other exterior stimuli. Nevertheless, when expressed as the full length receptor, the p55-TNF-R does not signal, unless stimulated by TNF. One must, therefore, assume that when activating the p55-TNF-R, TNF actually overcomes some inhibitory mechanisms, which prevent spontaneous association of the intracellular domains, and this inhibition is due to the linkage of the p55-IC to the rest of the receptor molecule. The inhibition may be due to the orientation imposed on the intracellular domain by the transmembrane and extracellular domain, to association of some other proteins with the receptor or perhaps just due to restriction of the amounts of receptors that are allowed to be placed in the plasma membrane. Of note, this control mechanism should be rather effective, since according to

some estimations, the binding of even just one TNF molecule to a cell suffices for the triggering of its death.

Spontaneous signaling, independent of ligand can result in extensive derangement of the process controlled by this receptor. The best known example is the deregulation of growth factor receptors. Mutations due to which they start signaling spontaneously, for example those that cause them to aggregate spontaneously, play an important role in the deregulated growth of tumor cells. TNF effects, when induced in excess, are well known to contribute to the pathology of many diseases. The ability of free intracellular domains (p55ICs) of the p55-TNF-R to signal independently of TNF may contribute to such excessive function. It seems possible, for example, that some of the cytopathic effects of viruses and other pathogens result, not from their direct cytocidal function, but from proteolytic detachment of the intracellular domain of the p55-TNF-R and the resulting TNF-like cytotoxic effect.

In addition to the above-mentioned, the present inventors have also recently shown (see IL 111125) that a region within the p55IC, the so-called 'death domain' or p55DD, is primarily responsible for the self-association capability and hence the ligand-independent cell cytotoxicity. Further, the present inventors have also disclosed the self-association capability of the FAS-IC (see also IL 111125). Accordingly, these p55IC, p55DD and FAS-IC proteins and the DNA sequences encoding them are known and have been utilized for isolating and identifying additional IC-binding proteins (See Example 3 below).

As regards the cytotoxc activity of the intracellular domains of the p55 TNF-R and FAS-R (p55IC and FAS-IC) it has now also been further elucidated that both the p55IC, its 'death domain' (p55DD) and the FAS-IC are capable of a ligand-independent triggering of a cytocidal effect in HeLa cells. In this study, HeLa cells were transfected with expression vectors containing various constructs of either the full-length p55-TNF-R, portions thereof including the p55IC and p55DD or the FAS-IC. In one set of experiments HeLa cells were co-transfected with constructs containing the p55 TNF-R (p55-R) and the FAS-IC (for details of the constructs, their preparation, etc. see above and IL 111125). The results of this study are depicted in Fig. 4 (A and B), wherein in both Fig. 4A and B

the constructs used for transfecting the HeLa cells are shown schematically in the left hand panels; the results of the TNF or FAS receptor expression are shown graphically in the two middle panels (second and third panels from the left); and the results of transfected cell viability are shown graphically in the right hand panels. In Fig. 4A there is shown the results of transfected HeLa cells transiently expressing the full-length p55-R, p55-IC or parts thereof, or as a control, luciferase (LUC), in all cases using a tetracycline-controlled expression vector. In Fig. 4B there is shown the results of transfected HeLa cells transiently expressing FAS-IC alone or together with the p55-R, using a tetracycline-controlled expression vector. In the graphic representation of the results in Fig. 4A and B, the open bars represent cells transfected in the presence of tetracycline (1 μg/ml), which inhibits expression, and the closed bars represent cells transfected in the absence of tetracycline. TNF receptor expression was assessed 20h after transfection, both by ELISA using antibodies against the extracellular domain of the receptor (see left hand panels), and by determining the binding of radiolabeled TNF to the cells (middle panels). The cytocidal effect of the transfected proteins was assessed 48h after transfection. The data shown are from one of three experiments with qualitatively similar results in which each construct was tested in duplicate. The designation 'ND' in Figs. 4A and B means not determined. From the results shown in Figs. 4A and B it is apparent that expression of only the p55IC results in even greater cytotoxicity. Significant cytotoxicity also occurs when expressing just the death domain (p55DD). In contrast, expression of parts of p55IC lacking the death domain or containing only part thereof, had no effect on cell viability. Expression of the FAS-IC did not result in significant cytotoxicity, yet it significantly enhanced the cytotoxicity of co-expressed p55-R.

EXAMPLE 3 :

Additional proteins capable of binding to the intracellular domains of p55 TNF-R or FAS-R

Using the same approach and technology set forth in Example 1 above, four more proteins have been isolated and identified which are capable of binding to the p55IC or FAS-IC.

In Figs. 5-8 there is shown schematically the partial and preliminary nucleotide sequence of cDNA clones, called HF1, F2, F9 and DD11, respectively.

Clone HF1 is a human cDNA clone isolated by the screening of a HeLa cDNA library. As the so-called "bait" the FAS-IC was used to isolate this clone. The isolated HF1 clone has an approximate length of 1.8 - 1.9 Kb, of which 1683 bases/nucleotides have been sequenced and are depicted in Fig. 5. Analysis of the binding capability of the protein encoded by clone HF1 (HF1) has shown that it interacts well (i.e. binds strongly to) with ~~the human FAS-IC, human FAS-DD, murine FAS-IC and murine FAS-DD. HF1 however~~ does not bind to p55IC, p75IC, p55DD, CD40-IC (another receptor of the TNF/NGF superfamily), nor does it bind to the irrelevant (control) proteins SNF1, Lamin or cyclin D which were also included in the binding assay. Further, a search conducted in the 'Gene Bank' and 'Protein Bank' DataBases revealed that there is no sequence corresponding to that of HF1. Thus, HF1 represents a new FAS-IC-specific binding-protein.

Clones F2 and F9 were isolated by screening a murine (mouse) embryonic library using the murine FAS-IC as "bait". In Fig. 6 there is shown schematically the partial nucleotide sequence from the F2 cDNA that has been sequenced. In Fig. 7 there is shown schematically the partial nucleotide sequence of 1724 bases from the F9 cDNA that has been sequenced. Analysis of the binding capability of the protein encoded by clones F2 and F9 (F2 and F9, respectively) has shown that :

(a) F2 interacts strongly with human p55IC and p55DD and with murine FAS-IC, while it interacts weakly with non-relevant (control) proteins SNF1 and Lamin as well as relevant protein, human FAS-IC.

(b) F9 interacts strongly with human p55-IC and murine FAS-IC, while it interacts weakly with human FAS-IC (relevant protein) and irrelevant proteins SNF1 and Lamin.

(c) Neither F2 nor F9 interacted at all with human p75IC, pGBT9 (empty bait vector), or human CD-40.

Further, from 'Gene Bank' and 'Protein Bank' searches it was revealed that F2 and F9 represent new proteins.

Thus, F2 and F9 represent new proteins having binding specificity for both FAS-IC and p55IC.

Clone DD11 was isolated by screening a human HeLa library using the human p55DD as "bait". In Fig. 8 there is shown schematically the partial nucleotide sequence of 425 bases from the DD11 cDNA that has been sequenced.

The DD11 clone has an approx. length of 800 nucleotides. The full length of the transcript is about 1.2 kb, the transcript having been probed using the clone. Analysis of the binding capability of the protein encoded by clone DD11 has shown that DD11 interacts strongly with the p55DD (a.a. 326-414) (see Fig. 4) and does not interact with deletion mutants of this domain, e.g. a.a. 326-404. DD11 also interacts with mouse and human FAS-IC and to some extent also with Lamin. DD11 does not interact at all with SNF1 nor with pGBT9 (empty bait vector). DD11 is also not found in the 'Gene Bank' and 'Protein Bank' databases. Thus DD11 represents a p55 IC (p55DD) and FAS-IC specific binding protein.

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CLAIMS

1. A DNA sequence encoding a protein capable of binding to one or more of the intracellular domains of one or more receptors belonging to the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily.

2. A DNA sequence according to claim 1, wherein said receptors are the TNF-Rs, p55 TNF-R or p75 TNF-R, or the FAS ligand receptor (FAS-R).

3. A DNA sequence according to claim 1 or claim 2, selected from the group consisting of:

   (a) a cDNA sequence derived from the coding region of a native TNF-R or FAS-R intracellular domain-binding protein;

   (b) DNA sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active TNF-R or FAS-R intracellular domain-binding protein; and

   (c) DNA sequences which are degenerate as a result of the genetic code to the DNA sequences defined in (a) and (b) and which encode a biologically active TNF-R or FAS-R intracellular domain-binding protein.

4. A DNA sequence according to any one of claims 1-3, encoding a p55 TNF-R intracellular domain (p55IC)-binding protein.

5. A DNA sequence according to claim 4 encoding a protein selected from the group comprising the herein designated proteins 55.1, 55.3, 55.11, F2, F9 and DD11.

6. A DNA sequence according to claim 5, selected from the sequences contained in the herein designated cDNA clones 55.1, 55.3, 55.11, F2, F9 and DD11.

7. A DNA sequence according to any one of claims 1-3, encoding a p75 TNF-R intracellular domain (p75IC)-binding protein.

8. A DNA sequence according to claim 7, encoding a protein selected from the group comprising the herein designated proteins 75.3 and 75.16.

9. A DNA sequence according to claim 8, selected from the sequences contained in the herein designated cDNA clones 75.3 and 75.16.

10. A DNA sequence according to claim 5 or claim 6 encoding the protein 55.1 having the amino acid sequence from amino acid residue 328 to residue 426 of the p55 TNF-R amino acid sequence.

11. A DNA sequence according to claim 5 of claim 6 encoding the protein 55.3 having the amino acid sequence from amino acid residue 277 to residue 426 of the p55 TNF-R amino acid sequence.

12. A DNA sequence according to claim 5 or claim 6, encoding the protein 55.11 comprising the sequence depicted in Fig. 1(a).

13. A DNA sequence according to claim 8 or claim 9 encoding the protein 75.3 comprising the sequence depicted in Fig. 1(b).

14. A DNA sequence according to claim 8 or claim 9 encoding the protein 75.16 comprising the sequence depicted in Fig. 1(c).

15. A DNA sequence according to any one of claims 1-3 encoding a FAS-R intracellular domain (FAS-IC)-binding protein.

16. A DNA sequence according to claim 15 encoding a protein selected from the group comprising the herein designated proteins HF1, F2, F9 and DD11.

17. A DNA sequence according to claim 16 selected from the sequences contained in the herein designated cDNA clones HF1, F2, F9 and DD11.

18. A DNA sequence according to claim 16 or 17 encoding any one of the proteins HF1, F2, F9 and DD11, comprising the sequences depicted in any one of Figs. 5-8, respectively.

19. A protein or analogs and derivatives thereof encoded by a sequence according to any one of claims 1-18, said protein, analogs and derivatives being capable of binding to one or more of the intracellular domains of one or more TNF-Rs or FAS-R.

20. A protein according to claim 19 selected from the group comprising the proteins 55.1, 55.3. 55.11, 75.3, 75.16, HF1, F2, F9 and DD11, and biologically active analogs and derivatives thereof.

21. A vector comprising a DNA sequence according to any one of claims 1-18.

22. A vector according to claim 21 which is capable of being expressed in a eukaryotic host cell.

23. A vector according to claim 21 which is capable of being expressed in a prokaryotic host cell.

24. Transformed eukaryotic or prokaryotic host cells containing a vector according to any one of claims 21-23.

25. A method for producing the protein analogs or derivatives according to claim 19 or 20 comprising growing the transformed host cells according to claim 24 under conditions suitable for the expression of said protein, analogs or derivatives, effecting post-translational modifications of said protein as necessary for obtention of said protein and extracting said expressed protein, analogs or derivatives from the culture medium of said transformed cells or from cell extracts of said transformed cells.

26. Antibodies or active fragments or derivatives thereof, specific for the protein, analogs or derivatives according to claim 19 or claim 20.

27. A method for the modulation of the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R, comprising treating said cells with one or more proteins, analogs or derivatives selected from the group consisting of the proteins, analogs and derivatives according to claim 19 or claim 20 and a protein being the p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives thereof, all of said proteins being capable

of binding to the intracellular domain and modulating the activity of said TNF-R or FAS-R, wherein said treating of said cells comprises introducing into said cells said one or more proteins, analogs or derivatives in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more proteins, analogs or derivatives in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

~~28. A method according to claim 27 wherein said treating of said cells is by transfection~~ of said cells with a recombinant animal virus vector comprising the steps of :

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of a TNF-R- or FAS-R- carrying cell and a second sequence encoding a protein selected from the proteins, analogs and derivatives according to claim 19 or 20 and a protein being the p55IC, p55DD, FAS-IC or FAS-DD, analogs or derivatives thereof, said protein when expressed in said cells being capable of modulating the activity of said TNF-R or FAS-R; and

(b) infecting said cells with said vector of (a).

29. A method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or a FAS-R comprising treating said cells with antibodies or active fragments or derivatives thereof, according to claim 26, said treating being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, wherein when the IC-binding proteins of said cells are exposed on the extracellular surface, said composition is formulated for extracellular application, and when said IC-binding proteins are intracellular said composition is formulated for intracellular application.

30. A method for modulating the TNF or FAS-R ligand effect on cells carrying a TNF-R or FAS-R comprising treating said cells with an oligonucleotide sequence selected

from a sequence encoding an antisense sequence of at least part of the sequence according to any one of claims 1-18, and a sequence encoding the antisense sequence of p55IC, p55DD, FAS-IC or FAS-DD, said oligonucleotide sequence being capable of blocking the expression of at least one of the TNF-R or FAS-R intracellular domain binding proteins.

31. A method according to claim 30 wherein said oligonucleotide sequence is introduced to said cells via a virus of claim 28 wherein said second sequence of said virus ~~encodes said oligonucleotide sequence.~~

32. A method for treating tumor cells or HIV-infected cells comprising:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein that is capable of binding to a specific tumor cell surface receptor and a sequence encoding a protein selected from the proteins, analogs and derivatives of claims 19 and 20 and the p55 TNF-R intracellular domain (p55IC), its 'death domain' (p55DD), the intracellular domain of FAS-R (FAS-IC), or its 'death domain' (FAS-DD), or a biologically active analog or derivative thereof, said protein, when expressed in said tumor cell being capable of killing said cell; and

(b) infecting said tumor cells with said vector of (a).

33. A method for modulating the TNF or FAS-R ligand effect on cells comprising applying the ribozyme procedure in which a vector encoding a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding a protein according to claim 19 or 20 or a mRNA sequence encoding p55IC, p55DD, FAS-IC or FAS-DD, is introduced into said cells in a form that permits expression of said ribozyme sequence in said cells, and wherein when said ribozyme sequence is expressed in said cells it interacts with said cellular mRNA sequence and cleaves said mRNA sequence resulting in the inhibition of expression of said protein or said p55IC, p55DD, FAS-IC or FAS-DD in said cells.

34. A method for isolating and identifying proteins, factors or receptors capable of binding to the intracellular domain binding proteins according to claim 19 or 20, comprising applying the procedure of affinity chromatography in which said protein according to claim 19 or 20 is attached to the affinity chromatography matrix, said attached protein is brought into contact with a cell extract and proteins, factors or receptors from cell extract which bound to said attached protein are then eluted, isolated analyzed.

~~35. A method for isolating and identifying proteins, capable of binding to the intracellular~~ domain binding proteins according to claim 19 or 20, comprising applying the yeast two-hybrid procedure in which a sequence encoding said intracellular domain binding protein is carried by one hybrid vector and sequence from a cDNA or genomic DNA library are carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said intracellular domain binding protein.

36. A pharmaceutical composition for the modulation of the TNF- or FAS-R ligand-effect on cells comprising, as active, ingredient a protein according to claim 19 or 20, or the protein p55IC, p55DD, FAS-IC or FAS-DD, its biologically active fragments, analogs, derivatives or mixtures thereof.

37. A pharmaceutical composition for modulating the TNF- or FAS-R ligand- effect on cells comprising, as active ingredient, a recombinant animal virus vector encoding a protein capable of binding a cell surface receptor and encoding a protein according to claim 19 or 20, or the protein p55IC, p55DD, FAS-IC or FAS-DD, its biologically active fragments or analogs.

38. A pharmaceutical composition for modulating the TNF or FAS-R ligand effect on cells comprising as active ingredient, an oligonucleotide sequence encoding an anti-sense sequence of the sequence according to any one of claims 1-18.

39. A method for isolating and identifying a protein capable of binding to the intracellular domains of TNF-Rs or FAS-R comprising applying the procedure of non-stringent southern hybridization followed by PCR cloning, in which a sequence or parts thereof according to any one of claims 1-18 is used as a probe to bind sequences from a cDNA or genomic DNA library, having at least partial homology thereto, said bound sequences then amplified and cloned by the PCR procedure to yield clones encoding proteins having at least partial; homology to said sequences of claims 1-18.

For the Applicant

Henry Einav

Fig 1a 55.11 clone

```
         |  10      |  20      |  30      |  40        |  50      |  60      |  70      |  80
   1 ATTCGGGTGC AGCCCCAGCA GTCTCCAGCG GCGGCCCCCG  GCGGCACGGA CGAGAAGCCG AGCGGCAAGG AGCGGCGGGA 80
  81 TGCCGGGGAC AAGGACAAAG AACAGGAGCT GTCTGAAGAG  GATAAACAGC TTCAAGATGA ACTGGAGATG CTCGTGGAAC 160
 161 GACTAGGGGA GAAGGATACA TCCCTGTATC GACCAGCGCT  GGAGGAATTG CGAAGGCAGA TTCGTTCTTC TACAACTTCC 240
 241 ATGACTTCAG TGCCCAAGCC TCTCAAATTT CTGTGTCCAC  ACTATGGCAA ACTGAAGGAA ATCTATGAGA ACATGGCCCC 320
 321 TGGGGAGAAT AAGCGTTTtG cTGCTGACAT CATCTCCGTT  TtGGCCATGA CCATGAGTGG GGAGCGTGAG TGCCTCAAGT 400
 401 ATCGGCTAGT GGGCTCCCAG GAGGAATTGG CATCATGGGG  TCATGAGTAT GTCAGGCATC TGGCAGGAGA AGTGGCTAAG 480
 481 GAGTGGCAGG AGCTGGATGA CGCAGAGAAG GTCCAGCGGG  AGCCTCTGCT CACTCTGGTG AAGGAAATCG TCCCCTATAA 560
 561 CATGGCCCAC AATGCAGAGC ATGAGGCTTG CGACCTGCTT  ATGGAAATTG AGCAGGTGGA CATGCTGGAG AAGGACATTG 640
 641 ATGAAAATGC ATATgCAAAG GTCTGCCTTT ATCTCACCAG  TTGTGTGAAT tACGTGCCTG AGCCTGAGAA CTCAgCCCTa 720
 721 CTGCGTTGTG CCCTGGGTGT GTTCCGAAAG TTTACCCGCT  TCCCTGAAGC TCTGAGATTG GCATTGATGC TCAATGACAT 800
 801 GGAGTTGGTA GAAGACATCT TCACCTCCTg CAAGGATGTG  GTAGTACAGA AACAGATGGC ATTCATGCTA GGCCGGCATG 880
 881 GGGTGTTCCT GGAGCTGAGT GAAGATGTCC ACCAGTATGA  GGACCTGACA GAGATCAtgt ccaatgtaca gcacaacagc 960
 961 aacttcttgg ccttagctcg ggagctggac atcatggagc  ccaaggtgcc tgatgacatc tacaaaaccc acctagagaa 1040
1041 caacaggttt gggggcagtt gctcTCAGGT GGACTCTGCC  CGCATGAACC TGGCCTCCTC TTTTGGGAAT GGGTGGGGGA 1120
1121 ATGCAGCTTT TGGCCAAGAC AAGCTGCTAA CAGATGATGG  CAACAAATGG CTTTACAAGA ACAAGGACCA CGGAATGTTG 1200
1201 AGTGCAGCTG CATCTCTTGG GATGATTCTG CTGTGGGATG  TGGATGGTGG CCTCACCCAG ATTGACAAGT ACCTGTACTC 1280
1281 CTCTGAGGAC TACATTAAGT CAGGAGCTCT TCTTGCCTGT  GGCATAGTGA ACTCTGGGGT CCGGAATGAG TGTGACCCTG 1360
1361 CTCTGGCACT GCTCTCAGAC TATGTTCTCC ACAACAGCAA  CAcCATGAGA CTTGGTTCCA TCTTTGGGCT AGGCTTGGCT 1440
1441 TATGCTGGCT CAAATCGTGA AGATGTCCTA ACACTGCTGC  TGCCTGTGAT GGGAGATTCA AAGTCCAGCA TGGAGGTGGC 1520
1521 AGGTGTCACA GCTTTAGCCT GTGGAATGAT AGCAGTAGGG  TCCTGCAATG GAGATGTAAC TTCCACTATC CTTCAGACCA 1600
1601 TCATGGAGAA GTCAGAGACT GAGCTCAAGG ATACTTATGC  TCGTTGGCTT CCTCTTGGAC TGGGTCTCAA CCACCTGGGG 1680
1681 AAGGGTGAGG CCATCGAGGC AATCCTGGCT GCACTGGAGG  TTGTGTCAGA GCCAttCCGC AGTTTtGGCA ACACACTGGT 1760
1761 GGATGTGTGT GCATATGCAG GCTCTGGGAA TGTGCTGAAG  GTGCAGCAGC TGCTCCACAT TTGTAGCGAA CACTTTGACT 1840
1841 CCAAAGAGAA GGAGGAAGAC AAAGACAAGA AGGAAAAGAA  AGACAAGGAC AAGAAGGAAG CCCCTGCTGA CATGGGAGCA 1920
1921 CATCAGGGAG TGGCTGTTCT GGGGATTGCC CTTATTGCTA  TGGGGGAGGA GATTGGTGCA GAGATGGCAT TACGAACCTT 2000
2001 TGgCCACTTG CTGAGATATG GGGAGCCTAC ACTCCGGAGG  GCTGTACCTT TAGCACTGGC CCTCATCTCT GTTTCAAATC 2080
2081 CACGACTCAA CATCCTGGAT ACCCTAAGCA AATTCTCTCA  TGATGCTGAT CCAGAAGTTT CCTATAACTC CATTTTTgCC 2160
2161 ATGGGCATGG TGGGCAGTGG TACCAATAAT GCCCGTCTGG  CTGCAATGCT GCGCCAGTTA GCTCAATATC ATGCCAAGGA 2240
2241 CCCAAACAAC CTCTTCATGG TGCGCTTGGC ACAGGGCCTG  ACACATTTAG GGAAGGGCAC CCTTACCCTC TGCCCCTACC 2320
2321 ACAGCGACCG GCAGCTTATG AGCCAGGTGG CCGTGGCTGG  ACTGCTCACT GTGCTTGTCT CTTTCCTGGA TGTtCGAAAC 2400
2401 ATTATTCTAG GCAAATCACA CTATGTATTG TATGGGCTGG  TGGCTGCCAT GCAGCCCCGA ATGCTGGTTA CGTTTGATGA 2480
2481 GGAGCTGCGG CCATTGCCAG TGTCTGTCCG TGTGGGCCAG  GCAGTGGATG TGGTGGGCCA GGCTGGCAAG CCGAAGACTA 2560
2561 TCACAGGGTT CCAGACGCAT ACAACCCCAG TGTTGTTGGC  CCACGGGGAA CGGGCAGAAT TGGCCACTGA GGAGtTTCTT 2640
2641 CCTGTTACCC CCATTCTGGA AGGTTTTGTT ATCTTCGGAA  GAACCCCAAT TATGATCTCT AAGTGACCAC CAGGGGCTCT 2720
2721 GAACTGCAGC TGATGTTATC AGCAGGACAT GCATCCTGCT  GCCAAGGGTG GACACGGCTG CAGACTTCTG GGGGAATTGT 2800
2801 CGCCTCCTGC TCTTTTGTTA CTGAGTGAGA TAAGGTTGTT  CAATAAAGAC TTTTATCCCC AAGGTC                2866
         |  10      |  20      |  30      |  40        |  50      |  60      |  70      |  80
```

(b) 75.3

5'GAATTCGGCACGAGCGGCACGAGGACAGAGTGAGACTCTGTCTCTTAAAATAATAATA
AAAATAAAAATAAAATGTGGGGCCGGGCAAGGTGGCTCATGCCTGTAATCCCAGCACCTT
GGGAGGCTGAGGCAGGAGGATTGCCTAAGCCCAGGAGTTTGACATCAGCCTGGGCAACAT
GGTGAAACCCCATCTCTACAAAAAATGCAAAAATTAGCCAGGTGTGGTGGGTGTGCTCCT
ATAGTCTCAGCTACTCAGGAGGCTGAGGTAGAGGGGATCACCTGAGCCCAGGAAGTTTGG
AGGCTATAGTGAGCTGAAGACCCGCACCATTGCACGCCAGCCTGGAGCAAGAGACNCTGT
CTCCACATAAATAAATAAATAAATAAAAGTGGGGAACTTCTGTGTTAAGTCAGAAGGCAC
CACACAATTTGNATAGCCANCAACCATATTCAATACCCAATCTCTTTATTGCAATATAAG
TATTTGTAAACCCCTACACAAATATTCCCAAGAATAAGTTGGAATATAAATTACTATATC
AATCANCCAATAAAAATAAACACATACAGTATTTATTTCCTGTTGCTCCATATAAAGCTT
TGCTATTTCAATATAAAGCTTACCTAGTATGGTCATTTGAGCCTGAGCAGAGAATATGCC
CAAGCTCGTGCCGAATTC....GGCGNTCTGACTCTCTACTGAACCAAGACTGAATCAGA
GAGACTCGAGTGCNCTTATTTGATTAANCCCAAATTATTGAAACCTNTGATTTTTTCTGG
AGGNGGATGATAAAGATGTGAAAGTGTGATGAACAGTGTGTATCCCTACTCTTGATCCTG
GAACCAGACAAGCAAGAAGCTTTGATTGAAAGCCTATGTGAAAAGCTGGTCAAATTTCGC
GAAGGTGAACGCCCGTCTCTGAGACTGCAGTTGTTAAGCAACCTTTTCCACGGGATGGAT
AAGAATACTCCTGTAAGATACACAGTGTATTGCAGCCTTATTAAAGTGGCAGCATCTTGT
GGGGCCATCCAGTACATCCCAACTGAGCTGGATCAAGTTAGAAAATGGATTTCTGACTGG
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TGTAAGAAGAGTGATGCTGCTTCAAAAGTCATGGTGGAATTGCTCGGAAGTTACACAGAG
GACAATGCTTCCCAGGCTCGAGTTGATGCCCACAGGTGTATTGTACGAGCATTGAAAGAT
CCAAATGCATTTCTTTGTGACCACCTTCTTACTTTAAAACCAGTCAAGTTTGTGGAAGGC
GAGCTTATTCATGATCTTTTAACCATTTGTGTGAGTGCTAAATTGGCATCATATGTCAAG
TTTTATCAGAATAATAAAGACTTCATTGATTCACTTGGCCTGTTACATGAACAGAATATG
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GCCGTAAGAACTAAAATGGTCTACTGCAAAATTGATCAGACCCAGAGAAAAGTAGTTGTC
AGTCATAGCACACATCGGACATTTGGAAAACAGCAGTGGCAACAACTGTATGACACACTT
AATGCCTGGAAACAAAATCTGAACAAAGTGAAAAACAGCCTTTTGAGTCTTTCTGATACC
TGAGTTTTTATGCTTATAATTTTTGTTCTTTGAAAAAAAAGCCCTAAATCATAGTAAAAC
ATTATAAACTAAAAAAAAAAAAAAAAAAACTCGAG 3'

FIGURE 1(b)

(c) 75.16

5'GTCCGGTTTACTTTAACTTAGTTTTGCATAGTTCTAGTGCACGTGAAATTGAAAAGTTA
TTTCCCTTTAGCTGTGTTATTATAGAGCAGAAATTCTGTTTTTAAAAATTAGCCTAAGATA
TACTTGTTTTTGTAAAGAAAAATATTTAATGCTTGAACAAAATAAATTGGAGTTGGAGTAG
AATGTAGTTTGAGGAAATTTGCAGCTTCCAATGCCTCTG.....CAGAGGCATTGGAAGCT
GCAAATTTCCTCAAACTACATTCTACTCCAACTCCAATTTATTTTGTTCAAGCATTAAATA
TTTTTCTTTACAAAAACAAGTATATCTTAGGCTAATTTTTAAAAACAGAATTTCTGCTCTA
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TAAGTTAAAGTAAACCGGAC 3'

FIGURE 1(c)

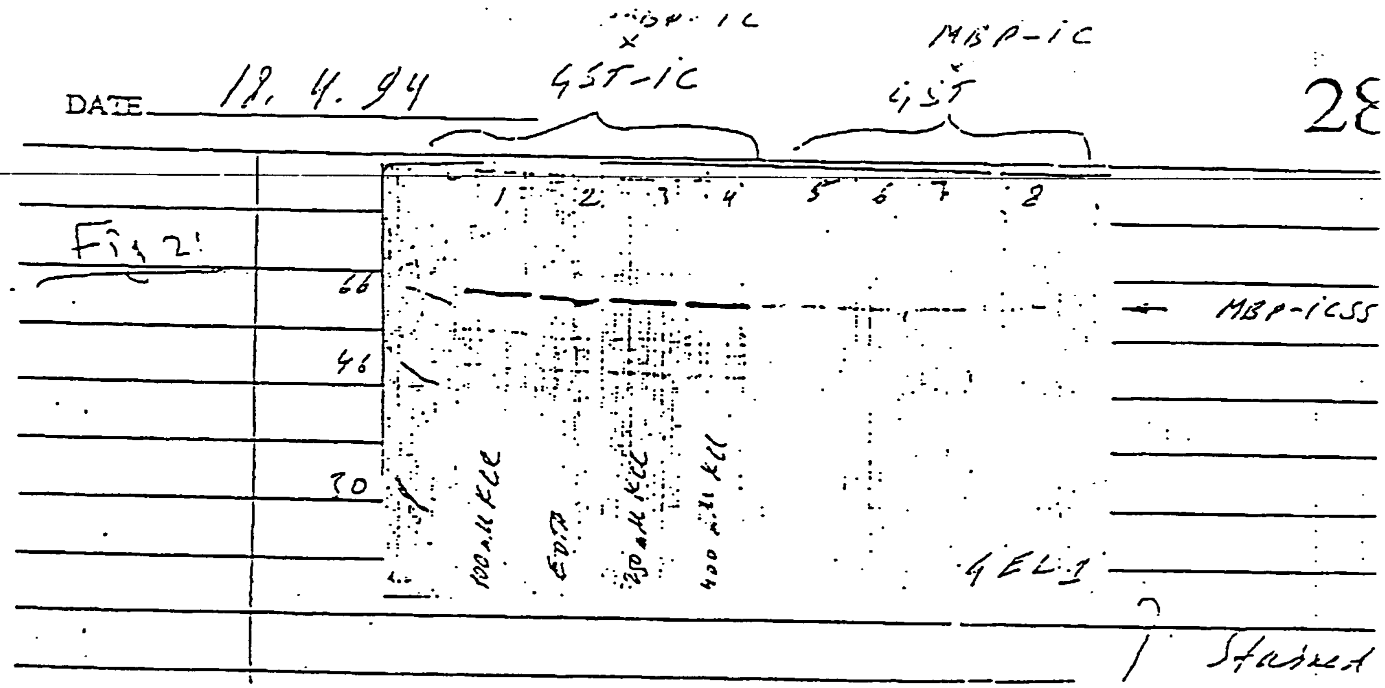
DATE 12. 4. 94
MBP-IC
GST-IC
MBP-IC
GST
28
Fig 2!
66
46
30
1 2 3 4 5 6 7 8
MBP-ICSS
GEL 1
Stained

FIGURE 2

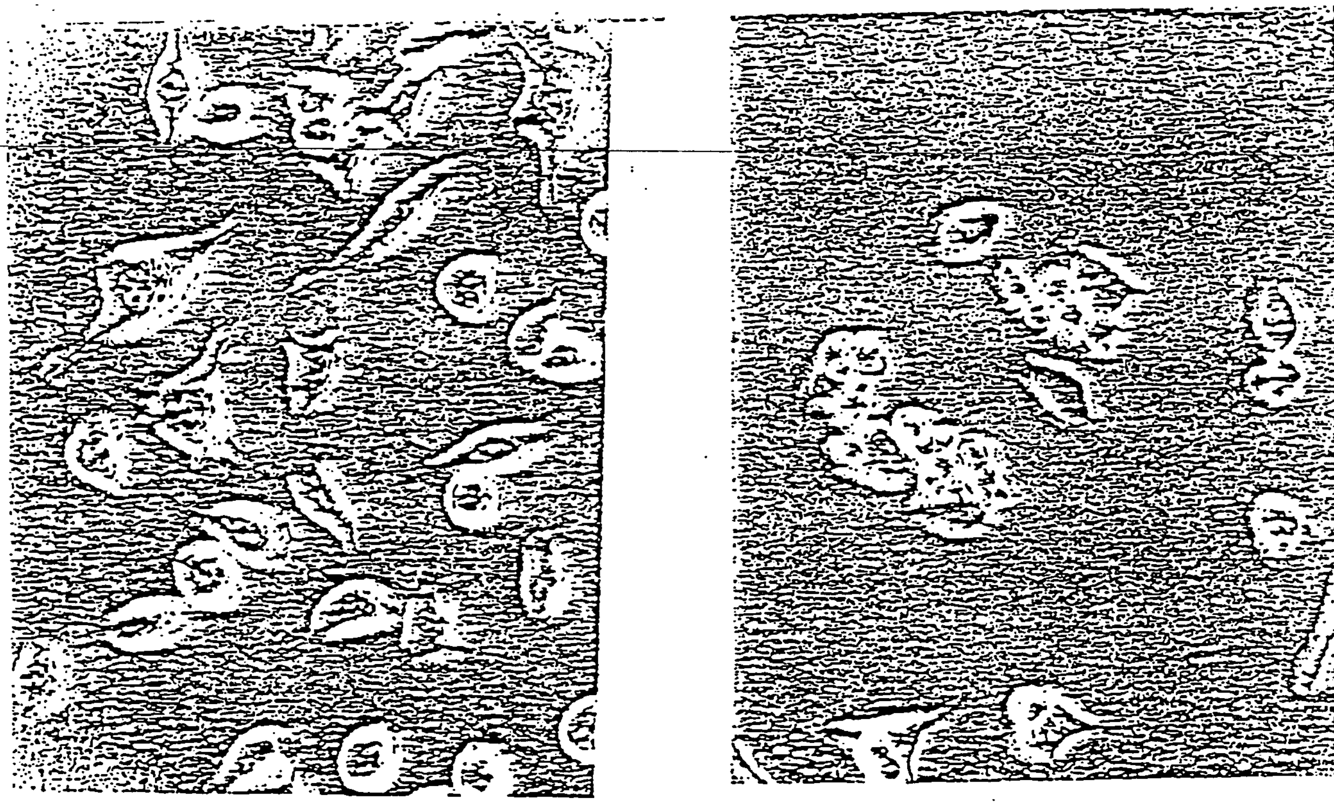

FIGURE 3

Fig 4

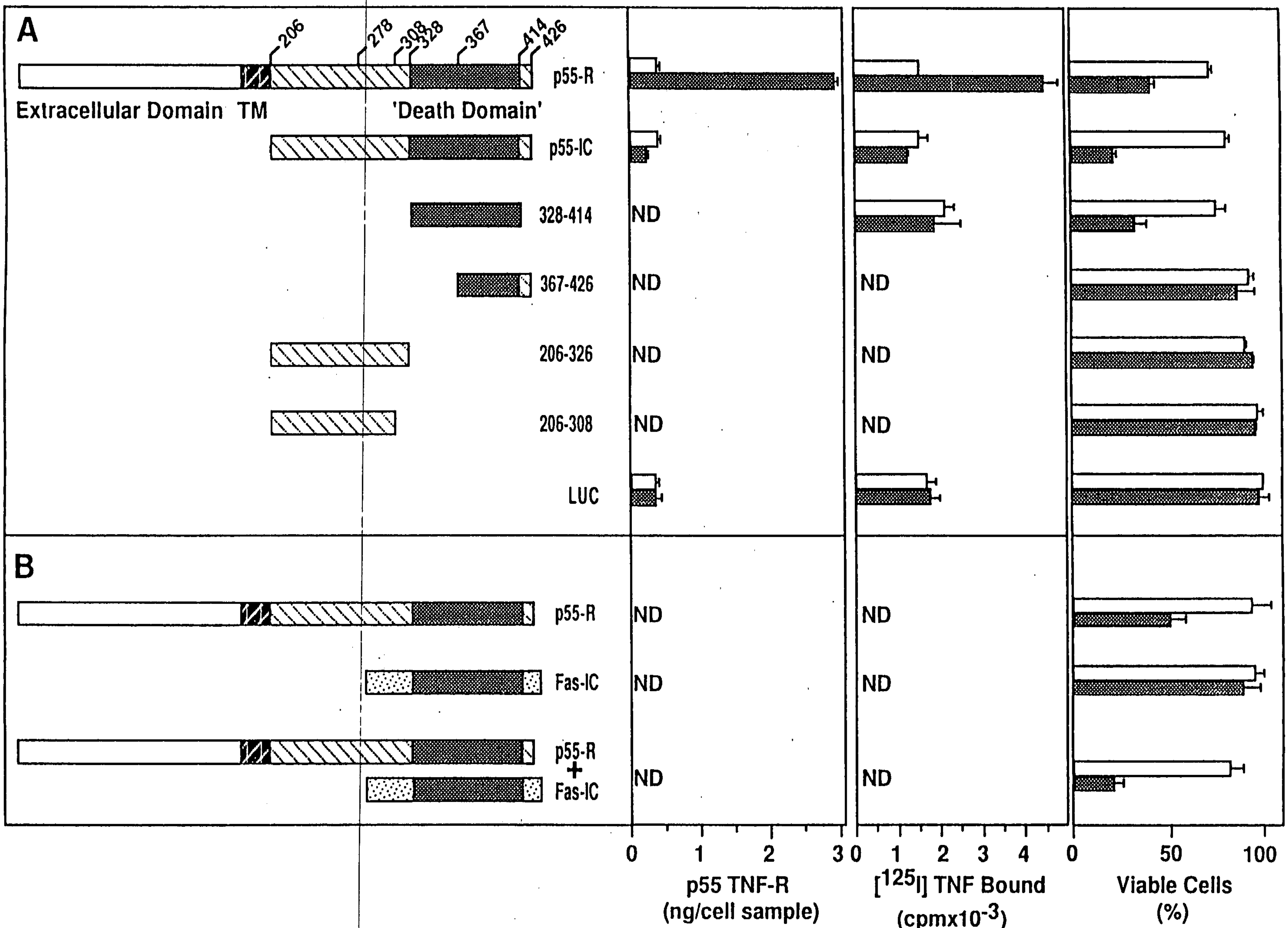
A
206
278
308
328
367
414
426
p55-R
Extracellular Domain TM
'Death Domain'
p55-IC
328-414
367-426
206-326
206-308
LUC
B
p55-R
Fas-IC
p55-R
+
Fas-IC
ND
0
1
2
3
p55 TNF-R
(ng/cell sample)
0
1
2
3
4
[125I] TNF Bound
(cpmx10-3)
0
50
100
Viable Cells
(%)

Fig.5 HF1 clone

```
          |  10      |  20      |  30      |  40       |  50      |  60      |  70      |  80
   1 AATTCGGCAC GAGGTGGAAT CCTTGGGCCG CTGGGCAAGC GGCGAGACCT GGCCAGGGCC AGCGAGCCGA GGACAGAGGG 80
  81 CGCGCGGAGG GCCGGGCCGC AGTCCCGGtC GtTTGCAGAC CCCGtCATGG ACCCGTTCCT GGTGCTGCTG CACTCGGTGT 160
 161 CGTCCAGCCT GTCGAGCAGC GAttTGACCG AGtTCAAGTT CCTATGCCTC GGGCGtGTGG TCAAGCGCAA GCTGGAGCGC 240
 241 GTGCAGAGCG GcCTAGACCT CTTCTCCATG CTGCTGGAGC AGAACGACCT GGAGCCCGGG CACACCGAGC TCCTGCGCGA 320
 321 GCTGCTCGCC TCCCTGCGGC GCCACGACCT GCTGCGGCGC GTCGACGACT TCGAGGCGGG GGCGGCGGcC GGGGCCGCGC 400
 401 CTGGGGAAGA AGACCTGTGT GCAGCATTTA ACGTCATATG TGATAATGTG GGGAAAGATT GGAGAAGGCT GGCTCGTCAG 480
 481 CTCAAAGTCT CAGACACCAA GATCGACAGC ATCGAGGACA GATACCCCCG CAACCTGACA GAGCGTGTGC GGGAGTCACT 560
 561 GAGAATCTgG AAGAACACAG AGAAGGAGAA CGCAACAGTG GcCCACCTGG TGGGGGCTCT CAGGTCCTGC CAGATGAACC 640
 641 TGGTGGCTGA CCTGGTACAA GAGGTTCAGC AGGCCCGTGA CCTCCAGAAC AGGAGTGGGG CCATGTCCCC GATGTCATGG 720
 721 AACTCAGACG CATCTACCTC CGAAGCGTCC TGATGGGCCG CTGCTTTGCG CTGGTGGACC ACAGGCATCT ACACAGCCTG 800
 801 GACTTTGGTT CTCTCCAGGA AGGTAGCCCA GCACTGTGAA GACCCAGCAG GAAGCCAGGC TGAGTGAGCC ACAGACCACC 880
 881 TGCTTCTGAA CTCAAGCTGC GTTTATTAAT GCCTCTCCCG CACCAGtCCG GTCtTGGGCC CTGCACAGAT ATTTCCATTT 960
 961 CTTCCTCACT ATGACACTGA GCAAGATCTT GTCTCCACTA AATGAGCTCC TGCGGGAGTA GTTGGAAAGT TGGAACCGTG 1040
1041 TCCAGCACAG AAGGAATCTG TGCAGATGAG CAGTCACACT GTTACTCCAC AGCGGAGGAG ACCAGCTCAG AGGCCCAGGA 1120
1121 ATCGGAGCGA AGCAGAGAGG TGGAGAACTG GGATTTGAAC CCCCGCCATC CTTCACCAGA GCCCATGCTC AACCACTGTG 1200
1201 GCGTTCTGCT GCCCCTGCAG TTGGCAGAAA GCATGTTTTG GTCCCATTTC CTTGGAGGCC ACCGGGACAG ACCTGGACAC 1280
1281 TAGGGTCAGG CGGGGTGCTG TGGTGGGGAG AGGCATGgCT GGGGTGGGGG TGGGGAGACC TGGTTGgCCG TGGTCCAGCT 1360
1361 CTTGGCCCCT GTGTGAGTTG AGTCTCCTCT CTGAGACTGC TAAGTAGGGG CAGTGATGGT TGCCAGGACG AATTGAGATA 1440
1441 ATATCTGTGA GGTGCTGATG AGTGATTGAC ACACAGCACT CTCTAAATCT TCCTTGTGAG GATTATGGGT CCTGCAATTC 1520
1521 TACAGTTTCT TACTGTTTTG TATCaAAATC ACTATCTTTC TGATAACAGA ATTGCCAAGG CAGCGGGATC TCGTATCTTT 1600
1601 AAAAAGCAGT CCTCTTATTC CTAAGGTAAT CCTATTAAAA CACAGCTTTA CAACTTCCAT ATTACaAAAA AAAAAAAAAA 1680
1681 AAA                                                                                     1683
          |  10      |  20      |  30      |  40       |  50      |  60      |  70      |  80
```

Fig.6 F2 clone

ATCAGTGTCACTACGGATAGTGATGACACTCACAGGAGGGCTGGGGTATCTgGAATGATGATT
GGCTGATGCTGGTCTTGGACAGGAACTAGGGAATTATAAGAAGATGTGGTACGAAGAGGACTAC
TCCCANCCAGAGAATAAACTTGAGAAGGCAGGACTTCCAGAGAGGATTTGGATGAAACTGGAGC
AGACTGCTTATTCTACTTTGAAGGGAGGGAACTAGACTGTTGTTGTCTGACAACATGGGCAACA
CCAACATTCAGAGGCTGAGCAGTNGcCAAGGNCACATGGTTGGTCAGCAAAGATGGCTGCTGCA
TAATAGTGCTGTACTGGTcGNCATGAGAGTGGGCATTCCCCAGTCAGCTAGCTGGTGGGCTGCT
CCCCAT...............................

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN...................................
.

cctctcagttatctctgttggagtagtcctcttcgtaccacatcttcttataattccctagttcctgtccaagaccagcatcagcCAA
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GAaGAaGAGGACAACAAATACAAGCCCAATAGCTCGAGCCTGAAGGcGAGGTCTAATGTCAT
CAGTTATGTCACTGTCAATGATTCTCCAGACTCTGACTCCTCCCTGAGCAGCCCACATTCCA
CAGCCACTCTGAGTGCTCTGcGGGGcAACAGTGGGACCCTTCTGGAGGGACCTGGCAGACC
TGCAGCAGATGGcATTGGCACCCGTACTATCATTGTACCTGAGCGGCCGC............
....

Fig.7   F9 clone

GGGAGCCTGTGCACCCCGATGTCACCATGAAGCCACTGCCCTTCTATGAAGTCTATGGGAGCTCATCCGAC
CCACCACCCTTGCGTCCACCTCCAGCCAGAGGTTCGAGGAAGCCCACTTCACCTTCGCGCTCACTCCCCAGC
AGCTGCAGCAGATTCTCACGTCCAGGGAGGTTATGCCAGGAGCCAAGTGTGATTACACCATACAAGTGCAGC
TCAGATTCTGTCTCTGTGAGACCAGCTGCCCTCAGGAGGACTATTTCCCCCCTAACCTCTTTGTTAAGGTTA
ATGGGAAACTCTGCCCCCTGCCGGGTTACCTCCCTCCAACCCAAGAATGGAGCTGAGCCCAAGAGGCCCAGC
CGTCCGATCAACATCACACCCTTGGCTCGACTCTCAGCCACTGTCCCCAACACCATCGTAGTTAATTGGGTC
ATCTTGAAGTTT.................................
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN....................................
tCCAACACCATCGTAGATAAATTGgTCATCTGAGTTTGGaCCGGAATTACTCCtTGTCCGTGTACCCTGGTG
AGGCAATTGACTGCAGGGACCCTTCTACACAAACTCAGAGCCAAGGGGATCCGGAATCCAGACCATTCCCGG
GCACTGATCAAGGAGAAACTGACTGCTGACCCCGACAGTGAAGTGGCTACTACAAGTCTCCGGGTGTCACTC
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GATGCTGcCCTTTATCTAcAGATgaaTGAGAAgAAGCCGACATGGACGTGTCCTGTGCGTGACAAGAAGGCT
CCCTATGAGTCGcTGATTATTGATGGTTTATTCATGGAAATTCTTAATTCCTGTTCGGATTGTGATGAGATC
CAGTTCATGGAAGATGGATCCTGGTGTCCGATGAAACCCAAGAAGGAGGcATCAGAGGTTTGCCCCCCGCCA
GGGTATGGGCTGGaTGGTCTCCAGTACAGCGCAGtCCAGGAGGGAATTCAGCCAGAGAGTAAGAAGAGGGTC
GAaGTCATTGACTTGACCATCGAAAGCTCATCAGATGAGGaggATTTGcCCCCCACCAAGAAGcACTGCCCT
GTCACCTCAGCGGtCATTCCAGCCCTTCCTGGAAGCAAAGGAGCCCTGAcCTCTGGTCACCAGcCATCCTCG
GTGCTGCGGAGCCCTGCAATGGGCACACTGGGcAGTGACTTCCTGTCTAGTCTCCCGCTACATGAGTACCCA
CCTGCCTTCCCACTGGGGGtTGACATCCAAGGTTTAGATTTTATTTTCTTTTCTTCAGACTGAGAGTCAGAA
TTACGGGCCTTCAGTTATCATTCG...................................
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN....................................
CCACTTCCTGGCCCACTGCCCCCAAACTGGGGACTCTCACCGcAAGCTCcAACTCCAGCgCCCCCTCCTGGT
CGTGTCAGCAGCATTGTGGCTCCTGGGAGCTCCTTGAGGGAAGGGCATGGAGGACCCCTGCCTTCAGGTC
CCTCTTTGACTGGCTGTCGGTCAGACGTCATTTCCTTGGACTGAGCTTTTTGGATTATGAAATCAATCTCC
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ATTGGCCAAAGGCCAGGCCAGAGCTTCATGGATACCTGCTTTTGGCCTTATCGCTGCCTAACAGGCCAGT
ACTCACAGGGTTAACATTTAACCTTTTTATGGTGGCCCG

Fig. 8 DD11 clone

....
AATTCGGCACGAGGTTGTGCTGTGGGGAAGGGAGAAGGATTTGTAAACCCCGGAGCGAGGTTCTGCTTACCC
GAGGCCGCTGCTGTGCGGAGACCCCCGGGTGAAGCCACCGTCATCATGTCTGACCAGGAGGCAAAACCTTCc
AACTGAGGACTTGGGGGATAAGAAGGAAGGTGAATATATTAAACTCAAAGTCATTGGACAGGATAGCAGTGA
GATTCACTTCAAAGTGAAAATGACAACACATCTCAAGAAACTCAAAGAATCATACTGTCAAAGACAGGGTGT
TCCAATGAATTCACTCAGGTTTCTCTTTGAGGGTCAGAGAATTGCTGATAATCATACTCCAAAAGAACTGGG
AATGGAGAAGAAAGATTGTGATTtGAaGTTTTATCAGGAACAAACGGGGGGTCATTCAACAGcTT.......
....